Information Disclosure Statement

The Applicants submit that the Information Disclosure Statement and Form PTO-1449 were submitted to the Patent Office on March 22, 2002.

Drawings

The Examiner asserted that the drawings in this application are objected to by the Draftsperson as informal. The Applicants acknowledge the objection, and will file a set of formal drawings under separate cover.

Specification

I. The Examiner objected to the disclosure because of the following alleged informalities:

a. On page 66, lines 26-27, large spaces occur between the words.

b. On page 70, lines 28-31, single spaced lines are employed.

The Applicants respectfully submit that the alleged informalities have been amended to eliminate further confusion. The Applicants hereby submit a replacement of page 66 to 119 of the specification, and respectfully request the insertion of the replacement into the application.

II. The Examiner objected to the use of trademarks in the application.

The Applicants have reviewed the specification but have not been able to identify any improper use of trademarks therein. The Applicants therefore respectfully request that the Examiner specifically point out any improper trademark usage.

Double Patenting

The Examiner rejected claims 11-14 under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over claims 1-20 of U.S. Application No. 09/513,783.

The Applicants acknowledge the rejection and will consider filing a terminal disclaimer once the claims are otherwise allowable.

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Claim rejections under 35 U.S.C. §103(a)

The Examiner rejected claims 11-14 under 35 U.S.C. §103(a) for allegedly being unpatentable over Taylor et al.(Optical Diagnostics of Living Cells and Biofluids, 2678: 15-27, 1996, "Taylor(96)") or Taylor et al. (American Scientist, 80:322-335, 1992, "Taylor(92)") in view of Hendzel et al. (Chromosoma, 1997, 106:348-360, "Hendzel"). The Applicants respectfully traverse the rejection.

In order to establish a *prima facie* case of obviousness the Examiner must establish three criteria: 1) a suggestion or motivation found within the prior art or within the knowledge of one of skill in the art to combine or modify the references; 2) a reasonable expectation of success; and 3) the prior art references alone or in combination must teach or suggest *all* the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art and not based on applicant's disclosure. MPEP § 706.02(j). Furthermore, when applying 35 U.S.C. §103 as set forth in *Graham v. John Deere*, the following tenets of patent law must be adhered to:

(A) the claimed invention must be considered as a whole;

(B) the references must be considered as a whole and must suggest the desirability and thus the obviousness of making the combination;

(C) the references must be viewed without the benefit of impermissible hindsight vision afforded by the claimed invention; and

(D) reasonable expectation of success is the standard with which obviousness is determined.

MPEP 2141. *Hodosh v. Block Drug Co., Inc.*, 786 F.2d 1136, 1143 n.5, 229 USPQ 182, 187 n.5 (Fed. Cir. 1986).

Claim 11 of the instant application recited an automated method for cell based toxin detection and organ localization comprising, providing an array of locations containing at least a first cell type and a second cell type, wherein the first and second cell types are not contained on the same location and are derived from different organ types, wherein each cell type comprises at least one luminescent reporter molecule, wherein the localization, distribution, structure, or activity of the at least one luminescent reporter is altered by a toxin to be detected; contacting the cells with a test substance;

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imaging and scanning the cells to obtain luminescent signals from the luminescent reporter molecule in the cells, converting the luminescent signals to digital data; and utilizing digital data to measure the localization, distribution, structure or activity of the luminescent reporter molecule on or in the cells, wherein a change in the localization, distribution, structure or activity of the luminescent reporter molecule indicates the presence of a toxin and an organ localization of the toxin. Claim 12 recites the use of a third cell type, wherein the first, second, and the third cell types are derived from different organ types. Claims 13 and 14 further recite that one or more cell types comprise at least two or at least three luminescent reporter molecules, whereas claim 15 recites that all cell types comprise at least two luminescent reporter molecules. Claim16 recites that the first luminescent reporter molecule is a detector, which detects the presence of a toxin, and the second luminescent reporter molecule is either a classifier or an identifier, which, in addition to detecting the presence of a toxin, identify a cell pathway affected by the toxin or a specific toxin or group of toxins, respectively. Furthermore, claim 17 recites that the second luminescent reporter molecule is a classifier, and the digital data derived from the classifier is used to select an identifier for identification of the specific toxin or group of toxins.

The Examiner admits that Taylor(96) and Taylor(92) fail to teach multiple cell analysis comprising two or three cell types with respect to toxin. The Applicants further note that Taylor(96) and Taylor(92) do not teach a method for toxin detection, nor do the cited references teach a method for toxin detection and organ localization comprising providing an array of locations containing two or three cell types derived from different organs, and analyzing the change in the localization, distribution, structure or activity of the luminescent reporter molecule to detect the presence of a toxin, to identify the pathway affected by the toxin, or to identify a specific toxin or group of toxins present in the test substance, and to characterize the organ localization of the toxin.

The Applicants respectfully submit that the Examiner's assertion that the example of using more than one tagging molecule in Taylor(92) "...meeting the limitation of dual luminescent reporters wherein one is a detector and the other is a classifier" is inaccurate. The cited portion of Taylor(92) teaches a proximity indicator. The present claims, in contrast, recite a detector that detects the presence of a toxin, and a classifier that, in

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addition to detecting the presence of a toxin, identifies a pathway affected by the toxin. Thus, Taylor(92) clearly does not anticipate, make obvious, or suggest the use of detectors and classifiers for toxin localization and organ distribution methods.

The deficiencies in Taylor(96) and Taylor(92) are not cured by Hendzel, which is asserted by the Examiner to describe the relationship between H3 phosphorylation and mitotic chromosome condensation in several mammalian cell lines. Hendzel does not teach or suggest, either alone or in combination with the cited Taylor references, a method for toxin detection, nor do the cited references teach a method for toxin detection and organ localization comprising providing an array of locations containing two or three cell types derived from different organs, and analyzing the change in the localization, distribution, structure or activity of the luminescent reporter molecule to detect the presence of a toxin, to identify the pathway affected by the toxin, or to identify a specific toxin or group of toxins present in the test substance, and to characterize the organ localization of the toxin.. Thus, the Examiner has not met step (3) in the test for establishing a *prima facie* case of obviousness, as the prior art references alone or in combination do not teach or suggest *all* the claim limitations.

Furthermore, the Examiner has not met the requirements of MPEP 2141, which dictate that the Examiner demonstrate that the cited references suggest the desirability and thus the obviousness of making the combination. This requirement is elaborated in MPEP 2143.01: "Obviousness can only be established by combining or modifying the teachings of the prior art to produce the claimed invention where there is some teaching, suggestion, or motivation to do so found either explicitly or implicitly in the references themselves or in the knowledge generally available to one of ordinary skill in the art." (MPEP 2143.01) It is also stated in MPEP 2143.01 that: "The mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination."

There is no motivation or suggestion in Taylor(96), Taylor(92) and Hendzel to combine these references, nor does the Examiner assert that there is any motivation or suggestion in the cited references of any desirability to combine these references. The Examiner simply implies that it would have been obvious to one of ordinary skill in the art at the time the invention was made to combine the references. However, "The level of

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skill in the art cannot be relied upon to provide the suggestion to combine references." Al-Site Corp. v. VSI Int'l Inc., 174 F.3d 1308, 50 USPQ2d 1161 (Fed.Cir.1999) MPEP 2143.01.

In summary, the combination of Taylor(96), Taylor(92) and Hendzel do not teach or suggest all the claim limitations of the current invention. Furthermore, there is no motivation or suggestion in Taylor(96), Taylor(92) or Hendzel to combine the references. Therefore, the teachings of Taylor(96) and Taylor(92) in view of Hendzel do not make obvious the invention of the instant application. The Applicants respectfully request that the Examiner reconsider and withdraw the rejection of claims 11-14 under 35 U.S.C.§103(a).

CONCLUSION

Based upon the above arguments, the Applicants respectfully submit that the claims are ready for allowance. If the Examiner believes that a telephone or personal interview would expedite prosecution of the instant application, the Examiner is invited to call the undersigned attorney at (312) 913-2106.

Respectfully submitted,
McDonnell Boehnen
Hulbert & Berghoff

Date: 9/24/02

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APPENDIX A. MARKED VERSION OF AMENDMENTS

In the specification
Please replace the paragraph on page 1 lines 4-9 with the following paragraph:

This application claims priority to U.S. Provisional Patent Application Serial Nos. 60/145,757 filed July 27, 1999; is a continuation-in-part of U.S. Patent Application S/N 09/540,862, filed March 31, 2000, which is a continuation in part of U.S. Patent Application S/N 09/401,212 filed September 22, 1999; which is a continuation in part of 08/865,341 filed May 29, 1997, **now U.S. Patent No. 6,103,479**; and is a continuation in part of U.S. Patent Application Serial No. 09/513,783 filed February 25, 2000, **now U.S. Patent No. 6,416,959**.

Please replace the paragraph on page 66, lines 24-29 with the following paragraph:

In a more preferred embodiment, the aminosilane is selected from the group consisting of methoxy or ethoxy silanes, which include but are not limited to trimethoxysilylpropyldiethylenetriamine, trimethoxysilylethylenediamine, aminopropyl-triethoxysilane, trimethoxyaminopropylsilane, or chlorosilanes such as trichlorosilylethylenediamine, aminopropyltrichlorosilane. In a most preferred embodiment, the amino silane is trimethoxysilylpropyldiethylenetriamine.

Please replace the paragraph on page 80, lines 11-21 as originally filed (now page 80 line 21-page 81, line 2) with the following paragraph:

Thus, in a preferred embodiment, the present invention results in a substrate with multiple types of differentiated cell types arranged in a pre-determined manner. The number of different cell types that can be arrayed is limited only by the differentiation potential of the stem cell, since the various cell binding locations can be individually addressed with differentiating agents, using devices including, but not limited to microspotters ~~(Cartesian Technologies[TM], Hewlett Packard[TM], Genetic MicroSystems[TM])~~, and fluid delivery system such as, but not limited to those disclosed herein, and in U.S. Patent Nos. 5,858,188; and 6,007,690. Selective addressing of the stem cells with differentiating agents enables controlled differentiation into the progeny

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of choice. In a preferred embodiment, the fluid delivery system of the present invention is combined with the patterned cell substrate to produce a microfluidic cassette, which can deliver differentiating compounds to the patterned undifferentiated stem cells.

Please replace the paragraph on page 87, lines 1-4 as originally filed (now page 87, lines 15-18) with the following paragraph:

The use of a fluid delivery system in the method, including but not limited to that disclosed above, or the use of automated precision instruments such as microspotters (~~Cartesian Technologies[TM], Hewlett Packard[TM], Genetic MicroSystems[TM]~~), permits the delivery of specific cell binding locations with a differentiating agent of choice.

In the claims
Please insert the following new claims:

15.(New) The method of claim 11, wherein the at least first cell type and the at least second cell type further comprise at least a second luminescent reporter molecule; wherein the localization, distribution, structure, or activity of the at least second luminescent reporter molecule is altered by a toxin to be detected.

16.(New) The method of claim 15, wherein the at least one luminescent reporter molecule is a detector, wherein the detector detects a toxin present in the test substance; and the second luminescent reporter molecule is selected from the group consisting of a classifier or an identifier, wherein the classifier detects a toxin present in the test substance and identifies a cell pathway affected by a toxin present in the test substance, and the identifier detects the presence of a toxin present in the test substance and identifies a specific toxin or group of toxins present in the test substance.

17.(New) The method of claim 16, wherein the second luminescent reporter is a classifier, and the digital data derived from the classifier is used to select an identifier for identification of the specific toxin or group of toxins.

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amount of oxygen gas is introduced, and charged particles are evolved between parallel-plated electrodes resulting in the cleavage of the O_2 bond. After this cleavage, high-energy free radicals can insert themselves into the polymer backbone resulting in the formation of various oxygen moieties, among which are hydroxyl groups. (U.S. Patent No. 5,357,005; U.S. Patent No. 5,132,108)

As used herein, the bifunctional molecule comprises

(a) a hydroxyl-reactive electrophile, including but not limited to silanes, carboxymethyl groups, succinimides, succinimidyl succinates, benzotriazole carbonates, glycidyl ethers (or epoxides), oxycarbonylimidazoles, p-nitrophenylcarbonates, aldehydes, isocyanates, and tresylates; and

(b) a nucleophile, including but not limited to sulfhydryl groups, amine groups, hydroxyl groups, or proteins or fragments thereof, peptides, and synthetic ligands for cell surface receptors, wherein the nucleophile can bind to other molecules and/or cells.

In one embodiment, the bifunctional molecule comprises an organosilane, wherein silane is the electrophile, and the nucleophile includes, but is not limited to sulfhydryl groups, amine groups, hydroxyl groups, or proteins, peptides, and synthetic ligands for cell surface receptors, wherein the nucleophile can bind to other molecules and/or cells. As used herein, organosilanes fall into a larger class of molecules, which have the capability of forming self-assembled (SA) films. The general form of this molecule comprises R_nSiX_{4-n}, where n = 1,2, or 3; X = Cl, OCH_3, or OC_2H_5, and R is the nucleophile as described above.

In a preferred embodiment, the bifunctional molecule comprises an aminosilane, wherein silane is the electrophile that attaches to the surface hydroxyl groups, and an amine group is the nucleophile that can bind to other molecules and/or cells.

In a more preferred embodiment, the aminosilane is selected from the group consisting of methoxy or ethoxy silanes, which include but are not limited to trimethoxysilylpropyldiethylenetriamine, trimethoxysilylethylenediamine, aminopropyl-triethoxysilane, trimethoxyaminopropylsilane, or chlorosilanes such as trichlorosilylethylenediamine, aminopropyltrichlorosilane. In a most preferred embodiment, the amino silane is trimethoxysilylpropyldiethylenetriamine

As used herein, a cell adhesive molecule includes compounds that: (1) introduce charge; and/or (2) are polar; and/or (3) contain sulfur and/or amines; and/or (4) are capable of tethering cells or other cell binding moieties, such as proteins, peptides, and synthetic ligands for cell surface receptors, thereby creating a cell binding location.

As used herein, the term cell repulsive moiety includes compounds that are capable of directly inhibiting cell binding, or that bind to other moieties which inhibit cell binding to the location, including polyethylene glycol (PEG) and other oxygen-rich compounds, sugars, hydrogels, extremely hydrophilic surfaces, or extremely hydrophobic surfaces.

In all of these embodiments, the cell adhesive molecule and/or cell repulsive moiety can be applied to the substrate via solution or vapor phase deposition. In a preferred embodiment, vapor deposition of the cell adhesive molecule and/or cell repulsive moiety is utilized. For example, apor phase deposition of various silanes has been demonstrated. (Tripp et al., Langmuir 8:1120-1126 (1992); Moses et al., Analytical Chemistry 20:4 (1978) In most cases, rather than adding the sample to a solution of silane, a hydroxylated surface is placed in the presence of vaporized silane (achievable by traditional vacuum techniques). The reaction takes place at the surface and results in self-assembled monolayers similar to that of silane solution deposition.

A wider range of cell adhesive molecules and cell repulsive moieties can be used with vapor phase deposition, because a solvent is not needed. For example, many silane solvents would dissolve the polymeric substrate and destroy its optical quality. In this embodiment, the method circumvents the use of solvents altogether.

In another preferred embodiment, the cell repulsive moiety comprises an amine-reactive moiety, including but not limited to 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride)-activated polyethylene glycol (PEG), polyvinylpyrrolidone, polyvinylalcohol, or any other amine-reactive extremely hydrophilic compound such as sugars (mannitol) or PEG, where the amine-reactive part can include, but is not limited to, carboxymethyl groups, succinimides, succinimidyl succinates, benzotriazole carbonates, glycidyl ethers (or epoxides), oxycarbonylimidazoles, p-nitrophenylcarbonates, aldehydes, isocyanates, and tresylates; or any amine-reactive extremely hydrophobic compound such as tridecafluoro-1,1,2,2-tetrahydrooctyl groups (13f) where the amine-reactive part can include, but is not limited to carboxymethyl

groups, succinimides, succinimydil succinates, benzotriazole carbonates, glycidyl ethers (or epoxides), oxycarbonylimidazoles, p-nitrophenylcarbonates, aldehydes, isocyanates, and tresylates. In a most preferred embodiment, the amine-reactive cell repulsive moiety comprises tresyl chloride-activated polyethylene glycol ("tresyl-chloride activated-PEG").

The chemistry of the tresyl-activated PEG can be used to regulate surface hydroxyl, amine, or sulfhydryl groups. Tresyl chloride will allow stable linkages to be formed between the nucleophile and the initial hydroxyl, amine, or sulfhydryl group carrying carbon. In a preferred embodiment, PEG is attached to a tresyl group for reaction with surface aminosilane groups.

In these preferred embodiments, cell adhesive cues can be defined by the use of a stencil, which has no size constraints. Cell repulsive cues, which also can be defined by the stencil, are tethered to an aminosilane monolayer. The cell binding locations may optionally be coated with cell adhesive proteins, protein fragments, or peptides, and seeded with cells resulting in a patterned array of cells.

This hydroxylated substrate is contacted with a bifunctional molecule comprising an electrophile and a nucleophile. This modified substrate is contacted with a textured elastomeric substrate (herein referred to as a 'stencil'), such as rubber, polyurethanes and poly(dimethyl) siloxanes ("PDMS"), to form a hermetic seal between defined regions of the stencil and the modified substrate. In a preferred embodiment, the stencil comprises PDMS. These materials are quite affordable, providing a significant benefit over traditional UV photolithography methods that employ a costly, high energy laser apparatus. (U.S. Patent No. 5,077,085)

The stencil comprises a physical mask that enables physical protection of defined regions of the underlying substrate from the subsequent solution or vapor phase deposition of the cell repulsive or cell adhesive moiety. This disclosed method of using a 'physical mask' distinguishes itself from existing art that relies on the use of an 'optical mask' (Dulcey et al., Science 252:551 (1991) and U.S. Patent Nos. 5,965,305 and 5,391,463) or 'contact imprinting' (U.S. Patent Nos. 5,512,131 and 5,776,748). The use of optical masks for protecting or de-protecting defined regions of a substrate is limited to the use of photoactivatible chemistries and/or photolabile molecules. The use of 'contact imprinting' is limited to solution phase transfer of materials onto a surface while not enabling 'protection' or 'de-protection' of defined regions of the surface. Further, contact imprinting does not enable reproducible transfer of

controlled amounts of material onto the surface. The use of a 'stencil', as disclosed in this invention, allows for protection of a region of the substrate to enable modification of unprotected regions with solution or vapor phase chemistries not limited to photoreactive/photolabile molecules.

The present invention is not constrained to one particular kind of substrate. The tethering chemistry of the primary monolayer, or the organosilane, is such that it reacts with surface hydroxyl groups. These hydroxyl groups can be introduced on the surface of virtually any plastic and glass by low temperature plasma treatment. The secondary tethering chemistry, tresyl chemistry, can react with surface amines, hydroxyl, and sulfhydryls, making it possible to attach to a wider array of surface chemistry. The desired effect is also achievable with high density surface hydroxyl groups, (which may eliminate any silane treatment). (Dust, Macromolecules, 1990. 23:3742-3746; U.S. Patent No. 5,330,911) All of these benefits make the disclosed method of patterning on glass and plastics affordable, facile, and accurate.

The benign nature of the chemistry employed makes it attractive for biological applications, allowing the array to be prepared on glass and any thermoplastic and thermoset of choice including, but not limited to poly(styrene), poly(olefin), poly(dimethyl) siloxane (PDMS), poly(carbonate), poly(vinyl) chloride, poly(ethylene), poly(ethylene) terapthalate, Teflon, and fluoronated ethylene co-poly(propylene) (FEP). The present methods also have the ease and flexibility to be applied to polymeric and glass substrates using the same method. Plastics such as poly(styrene), acrylics, and poly(olefin) have benefits over glass, ceramics and metals because of their affordability, flexibility of shape and size, ease of engineering, durability, low cost and control over its optical quality. The plastics are easily obtained at a minimal cost, can be molded into almost any shape conceivable, and are durable.

The present methods for preparing a substrate for selective cell patterning are more reproducible than are methods that employ contact printing, because there is less opportunity for operator error. There is operator dependence when contact printing due to the subjectivity of applying the stamp to the substrate (force by which the stamp is depressed, amount of solution on the stamp) and so the results will vary. (U.S. Patent No. 5,776,748) The present method of using a stencil for masking while performing solution or vapor phase deposition of the cell

adhesive molecules and/or cell repulsive moieties is operator independent, thus providing a scalable and manufacturable process.

The instantly disclosed method of cell patterning has a marked advantage over prior thiol chemistry. Previous technology of contact printing with thiols not only introduces operator error, but also requires a thin layer of gold to be evaporated on the surface of the tissue culture substrate. Due to the high temperature involved with gold evaporation, most plastics cannot be used. Optical quality is constrained and fluorescence light is absorbed due to the added layer of gold, which reduces the quality of information gathered when conducting cell-based screening. In addition to a lower optical quality, there is a high cost associated with gold coating. Furthermore, silane linkages are covalent, and are not subject to degradation, as are thiols on gold, which degrade over time due to impurities and the fact that a thiol bond is coordinate and not covalent. The methods of the present invention permit cell patterning on an optically clear substrate and give the added option of control over the substrate, so that one has the freedom to choose the most superior affordable plastic or glass for optical quality.

In a particular embodiment of the present method, oxygen plasma is used to activate the surface in the case of poly(styrene) and poly(olefin), and acid washing is used to activate the surface in the case of glass. Both surfaces can be further incubated with a mildly acidic alcoholic solution of aminosilane featuring a primary amine on the terminating end of the tethered molecule. Following silane treatment, a stencil is applied to the substrate. An aqueous solution of tresyl chloride-activated PEG is applied to the substrate around the stencil resulting in regions of exposed amine, and regions of PEG in carefully controlled proximity to one another. After surface modification, the surface can be primed with cell adhesive proteins, protein fragments, or peptides to speed the cell adhesion process. (U.S. Patent No. 5,874,219)

In another aspect, the present invention provides novel patterned substrates for cell culture. In one aspect, the invention provides cell patterning substrates, comprising:

1. at least a first portion having a reactive surface to which a plurality of cell adhesive molecules are coupled;

2. and at least a second portion having an exposed surface to which a plurality of cell repulsive moieties are coupled; wherein the cell adhesive molecules are selected from the

group consisting of silanes, and wherein the cell repulsive moieties comprise tresyl-chloride activated poly(ethylene) glycol.

In a preferred embodiment, the silane comprises R_nSiX_{4-n}, where n = 1,2, or 3; X = Cl, OCH_3, or OC_2H_5; R = a nucleophile, including but not limited to sulfhydryl groups, amine groups, hydroxyl groups, charged groups, polar groups, or proteins, protein fragments, peptides, and synthetic ligands for cell surface receptors, wherein the nucleophile can bind to other molecules and/or cells. In a preferred embodiment, the silane is an aminosilane. In a more preferred embodiment, the aminosilane is selected from the group consisting of methoxy or ethoxy silanes, which include but are not limited to trimethoxysilylpropyldiethylenetriamine, trimethoxysilylethylenediamine, aminopropyltriethoxysilane, trimethoxyaminopropyl-silane, or chlorosilanes such as trichlorosilylethylenediamine, aminopropyltrichlorosilane. In a most preferred embodiment, trimethoxysilylpropyldiethylenetriamine is used.

In another embodiment, the substrate further comprises cell adhesive proteins, protein fragments, or peptides, including but not limited to fibronectin, laminin, collagen, vitronectin, osteopontin, RGD peptides, RGDS peptides, YIGSR peptides. The strength of cell adhesion to the cell adhesion promoters can be modified by varying the composition of the cell adhesive proteins, protein fragments, or peptides. In a further embodiment, the substrate further comprises cells bound to the cell binding locations, either directly or indirectly via cell adhesive proteins, protein fragments, or peptides. Any cell type may be used, including prokaryotic, eukaryotic, and archaebacterial cells.

The cell binding locations according to the various methods and substrates of the invention can be as small as the diameter of a single cell and as large as several hundred cell diameters. The distance between cell binding locations (i.e.: the cell repulsive locations) is cell size dependent, but is sufficiently large so that a cell cannot bridge the gap between cell binding locations (i.e.: 1 cell diameter), unless a particular application calls for interaction of cells in different cell binding locations.

In a further embodiment, the various cell patterning substrates are mated with a fluid delivery system to provide fluid and/or reagent flow to the cell binding location. In a preferred embodiment, the fluid delivery system is that described herein.

In another embodiment, the cell patterning substrate comprises a cell patterning substrate made by the methods of the invention, as disclosed above.

This aspect of the present invention may be better understood with reference to the accompanying preferred embodiments that are intended for purposes of illustration only and should not be construed to limit the scope of the invention, as defined by the claims appended hereto.

Materials and Methods:

Reagents and instrumentation that can be utilized in carrying out the methods of the invention include, but are not limited to, 60 and 35 mm petri dishes, microplates, thermoplastics, poly(olefin), plasma cleaner/sterilizer, digital convection gauges, trimethoxysilylpropyldiethylenetriamine, and 2,2,2-trifluoroethanesylphonyl-poly(ethylene)$_{5000}$ glycol.

Poly(styrene), poly(olefin), or other thermoplastic substrates such as poly(esters) and poly(ether) are oxygen plasma treated inside a plasma cleaner using the following method. Substrates are placed inside the glass tube chamber and the chamber is evacuated to a pressure of ~200 mtorr as indicated by a convection gauge. Oxygen is pulsed in through a regulation valve and the chamber is evacuated again to a pressure of ~200 mtorr. The above oxygen pulse is repeated 2 more times. After the last oxygen pulse, the gas is allowed to bleed constantly into the chamber, and the final equilibrium pressure (with the oxygen bleed valve on and the vacuum pump activated) should be ~300 mtorr. After the proper pressure is reached, the voltage switch is turned up to HI (100W) and the substrates are treated for 25 minutes.

Glass surfaces are activated using the following method. A 1M KOH solution is prepared in double distilled/deionized (DI) water. The glass surfaces are incubated for 10 minutes in 1M KOH. After 10 minutes, the substrates are rinsed 3 times in double DI water. Coverslips are soaked in HCl:MeOH (1:1) for 30 minutes. After the incubation, the coverslips are rinsed in double DI water, and transferred into a concentrated bath of sulfuric acid for 30 minutes, followed by 3 rinses with double DI water. The coverslips are then boiled in distilled water for 15 minutes, and the surfaces blown dry with a nitrogen gun.

Aminosilane treatment is the same for glass, poly(styrene), and poly(olefin). A 1% solution of trimethoxysilylpropyldiethylenetriamine is prepared in mildly acidified methanol (94% methanol, 5% water, and 0.004% glacial acetic acid) and incubated with the substrates for 15 minutes. Following silane treatment, the substrates are rinsed with methanol and baked in a 80°C oven for 30 minutes.

The PDMS stencil is applied to the aminated glass or poly(styrene) (this embodiment includes but is not limited to 50, 100, 200 micron and 500 micron spots). Pressure is applied until the stencil makes a tight seal.

Tresyl-PEG treatment is the same for glass and poly(styrene). After stencil application, a 0.12M sodium bicarbonate solution is prepared in water. A 25% solution of tresyl-PEG (by weight) is prepared in the bicarbonate. The solution is applied around the stencil and allowed to pool around the PDMS, resulting in the liquid touching only exposed aminated surface areas. The substrates are incubated for 4 hours.

Following PEG treatment, the surfaces are rinsed with the 0.12M sodium bicarbonate solution. The substrates are allowed to incubate for 2 hours and rinsed under a stream of PBS. The substrates ("partially active") can be stored for more than 30 days in a dry box before protein coating and cell incubation.

As used herein, the term "partially active" substrates are glass or plastic substrates chemically and/or texturally modified to yield a patterned array of protein binding locations separated by cytophobic domains.

Protein coating

1. The partially active substrates are incubated in a protein, protein fragment, or peptide solution, including but not limited to fibronectin, laminin, collagen, vitronectin, osteopontin, or fragments thereof, as well as RGD peptides, RGDS peptides, YIGSR peptides, at concentrations ranging from 1 μg/ml to 25 μg/ml, for 1-2 hours.

2. Post-incubation, the substrates are rinsed in a stream of PBS.

3. The protein/peptide coated substrates are lyophilized to preserve the protein/peptide structure. These "quasi-active" substrates can be stored in a dry box for >30 days. The silane linkages are covalent and not subject to degradation, as are thiols on gold,

which degrade over time due to impurities and the fact that a thiol bond is coordinate and not covalent. As used herein, the term "quasi-active" substrates are glass or plastic substrates chemically and/or texturally modified to yield a patterned array of protein or peptide-rich domains separated by cytophobic domains.

Cell Seeding

1. Cells are prepared for seeding by standard techniques.

2. Partially active or quasi-active substrates are re-hydrated in cell culture medium for 5 minutes.

3. The cells are incubated at any desired seeding density with the partially or quasi active substrate for 30 minutes to 2 hours (depending on the cell type) in complete cell culture media at 37°C and 5% CO_2.

4. The "fully active" substrates, arrayed with the cells, can then be used for cell screening assays. After cryopreservation or room temperature desiccation the fully active substrates can be stored long term. As used herein, the term "fully active" substrates are glass or plastic substrates chemically and/or texturally modified to yield a patterned array of cells separated by cytophobic domains. They can be derived from either partially or quasi-active substrates, although the use of quasi-active substrates is preferred.

Example 5. Patterned stem cell and differentiated cell substrates

Stem cells possess the intrinsic ability to: (1) undergo self renewal, or (2) produce differentiated progeny. Extrinsic factors (culture medium, growth factors, physico-chemical cues from the surrounding cellular milieu) mediate the developmental fate of stem cells. Tissue specific stem cells, also called determined stem cells, also exhibit pluripotency but not totipotency. Determined stem cells provide the ability to access a primary, partially committed cell that can be driven to either self-replication in culture, or selectively differentiated into a multitude of tissue specific progeny that in-vitro are close, genotypically and phenotypically, to the whole organism, in contrast to immortalized cells that are both genotypically and phenotypically far removed from their precursor cells in the organism.

The pharmacological relevance of using stem cell derived tissue-specific progeny over immortalized cells results from the genotypic and phenotypic match afforded by the former. As a new chemical compound goes from being identified as "active" in a primary drug screen, it must pass through numerous tests designed to assess its pharmacological profile. Indices of bio-relevance such as cytotoxicity, bioavailablity, and specificity are evaluated together with potency to generate this profile, and require the use of cells genotypically and phenotypically matched to the organism. This increases the probability that the compound will be therapeutically relevant when it gets to the clinic beyond the animal testing stage. The use of primary cells (hepatocytes, neurons, chondrocytes, myocytes, adipocytes) are well documented in secondary and tertiary testing. However, the difficulty in obtaining the proper cells (access or amount), and culturing them in sufficient quantity to cover assay capacity remains a major problem. The use of stem cell derived primary cells can afford a solution to both higher relevance and a source of primary cells.

Other work has focused on the production of spatially oriented neo-vascular capillaries from endothelial cells that are bound to cell adhesion promoters patterned on a substrate. (Rudolph et al., U.S. Patent No. 5,721,131) However, the resulting substrate contained only a single type of differentiated cell, because the method did not permit individually addressing the cell binding locations with differentiating reagents.

The present invention provides methods for preparing patterned cell substrates comprising a multitude of terminally differentiated cells from an ordered array of stem cells, as well as the substrates themselves. The use of these patterned cell substrates for drug discovery increases the confidence level and relevance of the in-vitro pharmacological screening data for extrapolation to in-vivo settings.

The methods and substrate of the present invention mimic events in developmental biology: formation of committed mature or terminally differentiated cells from stem cells using controlled delivery of differentiation factors (including, but not limited to extracellular matrix (ECM) derivatized substrates, autocrine/paracrine/endocrine factors, etc.). Prior technology teaches induction of cellular heterogeneity by "peppering" a cell substrate surface with a multitude of cell adhesive domains enabling selective adhesion of one or more immortalized, genotypically and phenotypically distinct cell types from solution. The limited number of "cell-

specific cell adhesive moieties" limits the number of distinct cell types attainable on a single substrate. Prior technology does not take advantage of the intrinsic ability of stem cells to differentiate into a multitude of differentiated progeny.

In contrast, the present invention teaches induction of cellular heterogeneity on any substrate of choice (ceramics, metals, polymers, composites etc.) by the controlled differentiation of population(s) of stem cells into a multitude of differentiated cells. The controlled differentiation enables creation of "patterns of mixed cell types" in any number of variations and geometry. The present invention also enables creation of organ specific and tissue specific cell substrates that are close in genotypic and phenotypic relevance to the organism of choice, as well as substrates that model in vivo interactions between tissue specific and/or organ specific cells. This enables creation of a cell based screening platform capable of providing information with greater relevance to the organism or systemic level.

The preparation cell substrate of the present invention requires providing a patterned stem cell substrate, comprising:

(a) a substrate surface;

(b) a plurality of cell binding locations on the surface, wherein a cell binding location comprises:

(1) one or more cell adhesive molecules; and

(2) a plurality of stem cells bound to the cell adhesive molecules; and

(c) a plurality of cell repulsive locations on the surface, wherein the cell repulsive locations comprise a cell repulsive moiety , wherein individual cell binding locations are separated by cell repulsive locations.

A single type, or multiple types of stem cells are bound to the cell binding locations in a patterned array. As used herein, the term "patterned stem cell substrate" means that the stem cells are arrayed on the substrate in a controlled pattern. The stem cells can comprise a single class of stem cells, or may comprise multiple stem cell types, in which case the positioning of the different stem cell types is also controlled.

As used herein, the term "stem cells" refers to any cell type that possesses the ability to produce at least one type of differentiated progeny. As such, stem cells include, but are not limited to cells capable of differentiation into any cell type and self-renewal (pluripotent cells); cells capable of differentiation into any cell type but not self-renewal (totipotent cells); cells capable of self-renewal and differentiation into many cell types (broad potential; multipotent stem cells), cells capable of limited self-renewal and differentiation into limited types of cells (progenitor cells); and cells that have committed to differentiation into a specific cell type, but have not yet completed differentiation (committed progenitor cells). (See, for example, Gage et al. Science 287:1433-1438 (2000).) In various embodiments, the stem cells are selected from the group consisting of neural stem cells, neural progenitor cells, glial progenitor cells, mesenchymal stem cells, hematopoietic stem cells, epithelial stem cells, hepatic stem cells, embryonic stem cells, or combinations thereof. Reference to specific classes of stem cells is provided below:

Hepatic stem cells are reviewed in Thorgeirsson, *FASEB J.* 10:1249 (1996). Examples of hepatic stem cells include, but are not limited to, SEC cells, oval cells, as well as cultured WB-F344 cells, L2039 cells, and RLEΦ13 cells (Coleman et al., *Am. J. Pathol.* 151:353 (1997); Omori et al., *Hepatology* 26:720 (1997); Fiorino et al., In Vitro Cell Dev. Biol. Anim. 34:247-258 (1998); Agelli et al., Histochem. J. 29:205-217 (1997); Brill et al., Proc. Soc. Exp. Biol. Med. 204:261-269 (1993)).

Neural stem and progenitor cells: Examples of neuronal stem and progenitor cells include, but are not limited to, NT-2 cells (Pleasure et al., J. Neuroscience 12:1802-1815 (1992)), and those described in Gage, Science 287:1433-1438 (2000); Gritti et al., *J. of Neuroscience* 16:1091-1100 (1996); Frederiksen et al., *Neuron* 1:439 (1988); Reynolds and Weiss, *Science* 255:1707 (1992); Davis and Temple, *Nature* 372:263 (1994); McKay, *Science* 276:66-71 (1997);. Vicario-Abejon et al., *Neuron* 15:105 (1995); Johe et al., *Genes and Develop.* 10:3129-3142 (1996); and U.S. Patent Nos. 5,824,489; 6,001,654; 6,033,906).

Glial progenitor cells: Examples of glial progenitor cells include, but are not limited to SNB-19 cells and oligodendrocytes that can be diferentiated in vitro (Raible et al., J. Neurosci. Res. 34:287-294 (1993); (Welch et al., In Vitro Cell. Dev. Biol.-Animal 31:610-616 (1995)).

Mesenchymal stem cells are pluripotent progenitor cells that possess the ability to differentiate into a variety of mesenchymal tissue, including bone, cartilage, tendon, muscle, marrow stroma, fat and dermis. Such stem cells include, but are not limited to, C2C12 cells (Teboul et al., J. Biol. Chem. 270:28183-28187 (1995); Nishimura et al., J. Biol. Chem. 273:1872-1879 (1998)); and cells such as those described in U.S. Patent Nos. 5,486,359; 5,827,740; 5,942,225; 6,022,540.

Hematopoietic stem cells refer to any hematopoietic pluripotent progenitor cells capable of giving rise to a variety of differentiated hematopoietic cell types. Cell types within this definition include, but are not limited to $CD34^+$ bone marrow mononuclear cells (BMMC) (Berardi, et al., *Blood* 86:2123-2129, 1995), PBSC (Fritsch, et al., *Bone Marrow Transplantation* 17:169-178, 1996), cobblestone area forming cells (CAFC) (Lemieux, et al., *Blood* 86:1339-1347, 1995) and 5-FU BM cells (Alcorn and Holyoake, *Blood Reviews* 10:167-176, 1996); U.S. Patent No. 5,807,744)

Epithelial stem cells refer to cells that are long-lived, relatively undifferentiated, have a great potential for cell division, and are ultimately responsible for the homeostasis of epithelium. Cells of this type include, but are not limited to, those described in U.S. Patent No. 5,556,783; U.S. Patent No. 5,423,778; Rochat et al., *Cell* 76:1063 (1994); Jones et al. *Cell* 73:713 (1993); Jones et al., *Cell* 80:83 (1995)) and Slack, Science 287:1431-1433 (2000).

The cell adhesive molecules of this aspect of the invention can comprise any compound that is capable of supporting stem cell adhesion to the substrate, such as those described above, including cell adhesion molecules, co-polymer blends of extracellular matrix proteins or protein fragments such as RGD-containing peptides, silanes or thiols. In one embodiment, aminosilanes, such as methoxy or ethoxy silanes as disclosed above , are used.

The cell repulsive moieties comprise moieties that are capable of directly inhibiting cell binding, or that bind to other moieties which inhibit cell binding to the cell repulsive location, including but not limited to polyethylene glycol (PEG) and other oxygen-rich compounds, sugars, hydrogels, extremely hydrophilic surfaces, or extremely hydrophobic surfaces, as described above.

The cell binding locations may also comprise inhibitors of uncontrolled differentiation of the stem cell, which can comprise any compound known in the art to prevent uncontrolled stem

cell differentiation. Such compounds include, but are not limited to self-assembled monolayers of thiols or silanes coupled to cell adhesive ligands, which are utilized to enable the creation of cell binding locations, while preventing their uncontrolled differentiation. Another example is the addition of protease thrombin to cultures of the Neuro2A neuroblastoma cell line, which inhibits differentiation of the cells into neurite-containing neuronal cells. (Gurwitz et al., Proc. Natl. Acad. Sci. 85:3440-3444 (1988)) Thus, the cell binding promoter can also serve as an inhibitor of uncontrolled differentiation. Alternatively, the cell binding locations are covered with a feeder layer of cells to inhibit uncontrolled stem cell differentiation.

The substrate of this aspect of the invention can be made of any material known in the art, including but not limited to plastics, glass, ceramics and metals. Preferably, such patterned substrates are made on commercially viable plastic substrates such as polystyrene or poly(olefin). In a preferred embodiment, the substrates possess 100 μm-500 μm diameter circular cell binding locations separated by edge-to-edge spacing ranging from 25 μm to 125 μm. The overall footprint on the substrate will match the format of 96, 384, and 1536 well microplates, and be approximately 150-fold smaller in surface area compared to 1536 (32 rows x 48 columns), 384 (16 rows x 24 columns), and 96 wells (8 rows x 12 columns) microplates.

In a further aspect, the present invention provides a patterned differentiated cell substrate comprising:

(a) a substrate surface;

(b) a first plurality of cell binding locations on the surface comprising:

(i) one or more cell adhesive molecule;

(ii) a first differentiated cell type;

(c) a second plurality of cell binding locations on the surface comprising:

(i) one or more cell adhesive molecule;

(ii) a second differentiated cell type; wherein the first and second differentiated cell types are arrayed on the substrate in a controlled pattern; and

(d) a plurality of cell repulsive locations on the surface, wherein the cell repulsive locations comprise a cell repulsive moiety , wherein individual cell binding locations are separated by cell repulsive locations.

As used herein, the term "patterned differentiated cell substrate" means that the differentiated cells are arrayed on the substrate in a controlled fashion, wherein the terminal differentiation is achieved post-patterning..

In one embodiment, the differentiated cells are derived from stem cells that were selectively differentiated on the substrate. In this embodiment, the stem cell type is selected from the group consisting of neural stem cells, neural progenitor cells, glial progenitor cells, mesenchymal stem cells, hematopoietic stem cells, epithelial stem cells, hepatic stem cells, embryonic stem cells, or combinations thereof. Alternatively, differentiation can be initiated prior to plating the cells (committed progenitor cells) on the substrate . One of skill in the art will be able to envision many permutations of this embodiment, including but not limited to the following:

1. Selective differentiation of a single stem cell type into two or more differentiated cell types in predetermined locations on a single substrate.

2. Selective differentiation of two or more stem cell types arrayed on a single substrate to generate two or more differentiated cell types.

3. Sequential, selective differentiation of a single or multiple stem cell types, whereby a single differentiated cell type is produced first, followed by selective differentiation of the stem cell type(s) in other cell binding locations.

Thus, in a preferred embodiment, the present invention results in a substrate with multiple types of differentiated cell types arranged in a pre-determined manner. The number of different cell types that can be arrayed is limited only by the differentiation potential of the stem cell, since the various cell binding locations can be individually addressed with differentiating agents, using devices including, but not limited to microspotters, and fluid delivery system such as, but not limited to those disclosed herein, and in U.S. Patent Nos. 5,858,188; and 6,007,690. Selective addressing of the stem cells with differentiating agents enables controlled differentiation into the progeny of choice. In a preferred embodiment, the fluid delivery system of the present invention is combined with the patterned cell substrate to produce a microfluidic

cassette, which can deliver differentiating compounds to the patterned undifferentiated stem cells.

In a preferred embodiment, the first and second differentiated cell types are cell types found in a single tissue, including but not limited to brain, vascular tissue, skin, pancreas, kidney, liver, lung, heart, intestine, and stomach.

In another preferred embodiment, the first and second differentiated cell types are cell types that are found in different tissues that interact in vivo, including but not limited to peripheral nerve-smooth and/or skeletal muscle; epithelial tissue-smooth muscle; vascular endothelium-smooth muscle; glial cells-endothelial cells of blood capillaries; adipose cells-axons of peripheral nerve cells and/or Schwann cells; and pancreatic B cells-pancreatic A cells.

In another preferred embodiment, the first and second differentiated cell types are selected from the group consisting of glial cells, neurons, adipocytes, smooth muscle cells, skeletal muscle cells, osteoblasts, chondrocytes, stromal cells, and myocytes.

In various most preferred embodiments, (a) the first differentiated cell type derived is a glial cell, and the second differentiated cell type is a neuronal cell, thus producing a cell substrate model for the brain; (b) the first differentiated cell type is an adipocyte and the second differentiated cell type is a muscle cell; (c) the first differentiated cell type a neuronal cell and the second differentiated cell type is a muscle cell.

In a further preferred embodiment, the patterned stem cell substrate or the patterned cell substrate is mated with a fluid delivery system chamber to form a cassette, wherein the fluid delivery system delivers reagents to the stem cells or differentiated cells, and comprises:

(i) a plurality of domains matching the cell binding locations on the surface of the substrate, and

(ii) microfluidic channels that supply reagents to the cell binding locations.

As used herein, "reagents" include differentiating agents, as well as cell culture medium and any cell culture supplements for cell culture, and test compounds for screening the effects of drug compound libraries and toxins on the cells.

In a most preferred embodiment, a single microfluidic channel supplies fluid to a single cell binding location on the substrate, to provide separate fluid flow to each cell binding location,

thereby permitting selective differentiation of the stem cells, and/or selective treatment of the stem cells or differentiated cells in one or more cell binding locations with a test agent of choice.

In another aspect, the present invention provides a patterned differentiated cell substrate made by a method of selective differentiation of patterned stem cells, wherein the method comprises:

(a) providing a patterned stem cell substrate, comprising

(1) a substrate surface;

(2) a first plurality of cell binding locations on the surface comprising

(i) one or more cell adhesive molecules;

(ii) a first differentiated cell type;

(3) a second plurality of cell binding locations on the surface comprising:

(i) one or more cell adhesive molecules ;

(ii) a second differentiated cell type; wherein the first and second differentiated cell types are arrayed on the substrate in a controlled pattern;

(b) selectively contacting the cell binding locations with differentiating agents to provide controlled differentiation of the stem cells into a progeny of choice, wherein the selective contacting produces a patterned differentiated cell substrate.

In a preferred embodiment, the patterned cell substrate is mated with a fluid delivery system as described above.

In further preferred embodiments of the above aspects, the stem cells and the resulting differentiated cells possess at least one luminescent reporter molecule, for use in cell screening assays to determine the distribution, environment or activity of the luminescent reporter molecules on or in the cells or within or between subcellular compartments of the cells, in response to a test agent of choice. A variety of such luminescent reporter molecules can be used in this aspect of the invention, including but not limited to those described in U.S. Patent No. 5,989,835; pending U.S. Patent Application Nos. 09/031,271 (filed February 27, 1998); 09/352,171 (filed July 12, 1999); 09/293,209 (filed April 16, 1999); 09/293,209 (filed April 16, 1999); 09/398,965 (filed September 17, 1999); 09/430,656 (filed October 29, 1999); 09/513,783

(filed February 24, 2000); Giuliano et al., Ann. Rev. Biophys. Biomol. Struct. 24:405-434 (1995); and Giuliano et al., Trends in Biotech. 16:135-140 (1998), all references incorporated by reference herein in their entirety.

The luminescent probes can be small molecules, labeled macromolecules, or genetically engineered proteins, including, but not limited to green fluorescent protein chimeras. As used herein, "luminescent" probes include fluorescent, luminescent, and chemiluminescent probes.

In another embodiment, only one of the differentiated cell types possesses a luminescent reporter molecule, and when an interaction occurs between the first and second differentiated cell types, only one of the cell types reports the interaction, via the luminescent reporter molecule.

The luminescently labeled reporter molecule may be expressed by or added to the cells either before, together with, or after contacting the cells with a test compound. For example, the reporter molecule may be expressed as a luminescently labeled protein chimera by transfected stem cells. Alternatively, the luminescently labeled reporter molecule may be expressed, isolated, and bulk-loaded into the stem cells, or the reporter molecule may be luminescently labeled after isolation. As a further alternative, the reporter molecule can be expressed by the stem cell, which is subsequently contacted with a luminescent label, such as a labeled antibody, that detects the reporter molecule.

Preferably, the luminescent reporter molecules in the first differentiated cell type are spectrally distinguishable from the luminescent reporter molecules in the second differentiated cell type.

The present invention also provides methods for selective stem cell differentiation, comprising providing a patterned stem cell substrate as disclosed above, and selectively contacting the cell binding locations with differentiating agents to provide controlled differentiation of the stem cells into the progeny of choice, wherein the selective contacting produces a patterned differentiated cell substrate. Any method known in the art for differentiating the stem cells into differentiated cells can be used. References providing conditions for differentiating the various stem cells into differentiated progeny can be found, for example, in the following references, which are incorporated by reference herein in their entirety:

Mesenchymal stem cells: U.S. Patent No. 5,827,740 (adipogenic differentiation); U.S. Patent No. 6,022,540 (osteogenic differentiation); U.S. Patent No. 5,942,225 (osteogenic, chondrogenic, tendenogenic, marrow stromal cell, and myogenic differentiation); Cuenda et al., J. Biol. Chem. 274:4341-4346 (1999) (C2C12 myogenic differentiation); Nishimura et al., J. Biol. Chem. 273:1872-1879 (1998) (C2C12 osteoblastic differentiation); Teboul et al., J. Biol. Chem. 270:28183-28187 (1995) (C2C12 adipogenic differentiation)

Neural stem and progenitor cells: U.S. Patent No. 5,824,489 (neurons and glia); U.S. Patent No. 6,001,654 (neurons and smooth muscle cells); U.S. Patent No. 6,033,906 (glial cells); Pleasure et al., J. Neurosci. 12:1802-1815 (1992) (NTera2 differentiation into neurons);

Glial and neural stem and progenitor cells: Welch et al., In Vitro Cell. Dev. Biol.-Animal 31:610-616 (1995) (SNB-19 glial differentiation); U.S. Patent No. 5,824,489 (neurons and glia).

Hematopoietic stem cells: Lawman et al., J. Hematother. 1:251-259 (1992); Huss, Stem Cells 18:1-9 (2000); Zhang et al., Blood 95:138-146 (2000); Zhang et al., Blood 92:118-128 (1998).

Hepatic stem cells: Brill et al., Proc. Soc. Exp. Biol. Med. 204:261-269 (1993); Brill et al., Dig. Dis. Sci., 44:364-371 (1999); Fiorino et al., In Vitro Cell Dev. Biol. Anim. 34:247-258 (1998).

In various preferred embodiments, the substrate is mated with a fluid delivery system as disclosed above, and the stem cells possess at least one luminescent reporter molecule that serves to identify the phenotype of the progeny of choice.

In these embodiments, the stem cells are contacted with differentiating agents, to effect differentiation of the transfected patterned cells into differentiated progeny, using the appropriate differentiating agents applied either homogeneously to the substrate (for single progeny) or selectively to specific cell binding locations (multiple progeny). Differentiation of a single stem cell type into different progeny, or of different stem cells into different progeny, permits the formation of substrates with cell binding locations that bear different cell types in any desired juxtaposition. In this way, simple to moderately complex models of cellular differentiation are used to prepare multicellular tissue-specific and organ-specific cell substrates for use in cell based analysis for drug discovery and life sciences.

In one model system, neuronal and glial stem cells are engineered to express fluorescent protein reporter molecules to measure the dynamics of their cytoskeletal proteins. The cytoskeleton has become a well-characterized and valid drug discovery target for which there are likely to be many lead compounds in the drug discovery pipeline at any one time. Each of the stem cell lines express spectrally distinct reporter molecules such that they can be patterned into separate locations within a cell array as well as be patterned together, (co-cultured) within the same location. Alternatively, the different stem cells may be patterned into separate locations, wherein the locations are in close enough proximity to permit interactions between the cells in the different locations. The latter two aspect allow the simultaneous measurement of drug responses of the two cell types in an organotypic context where the cells are allowed to interact as they would within the brain tissue of a living animal. Because the reporter molecules contained within each cell type are spectrally distinct, the platform detects and assigns function to each cell type within the co-culture.

In a second model system, a pluripotent cell line, mouse C2C12 cells, is engineered with a luminescent reporter molecule, patterned into microarrays, and differentiated into two cell types, skeletal muscle myocytes and adipocytes. In this case, the stem cells are engineered to express a luminescent reporter molecule of carbohydrate metabolic flux, including but not limited to reporters of the phosphorylation state of PFK-1 and PFK-2, the measurement of cellular ATP levels (energy charge), the ratio of oxidation-reduction co-factors such as NAD^{+}/NADH, and the concentration of the second messenger cAMP, that are measured both in time and in space within each cell type. For muscle and adipose cells, carbohydrate metabolism plays an important role in regulating the physiological function of each cell type; contraction and relaxation of the muscle cells and fat storage and mobilization in adipocytes. Therefore, this model system permits measurement of the effect of lead compounds on the same molecular pathway within two tissue types. Moreover, this multiple tissue type screening platform permits the efficient addressing of lead compound efficacy, specificity, and toxicology.

A third, more complex model system involves the co-cultivation of neuronal and skeletal muscle stem cells with both types being engineered to express spectrally distinct luminescent reporter molecules. The cells are patterned and differentiated on the substrate. The effect of lead compounds on the complex interaction of neurons and skeletal muscle cells is measured using

the luminescent reporter molecules engineered into each cell type. The co-cultivation of differentiated neuronal and muscle cells permits direct measurement of excitation-contraction coupling events and the effects that lead compounds have on these events.

These approaches can be generalized to interactions between other tissue types and interactions between multiple cell types within an organ, including but not limited to peripheral nerve-smooth or skeletal muscle; epithelial tissue-smooth muscle; vascular endothelium-smooth muscle; glial cells-endothelial cells of blood capillaries; adipose cells-axons of peripheral nerve cells and Schwann cells; and pancreatic B cells-pancreatic A cells.

The present invention further provides methods for cell based screening, wherein the stem cells and/or differentiated cells possess at least one luminescent reporter molecule, for use in cell screening assays to determine the distribution, environment or activity of the luminescent reporter molecules on or in the cells or within or between subcellular compartments of the cells, in response to a test agent of choice. A variety of such luminescent reporter molecules can be used in this aspect of the invention, including but not limited to those described in U.S. Patent No. 5,989,835; pending U.S. Patent Application Nos. 09/031,271 (filed February 27, 1998); 09/352,171 (filed July 12, 1999); 09/293,209 (filed April 16, 1999); 09/293,209 (filed April 16, 1999); 09/398,965 (filed September 17, 1999); 09/430,656 (filed October 29, 1999); 09/513,783 (filed February 24, 2000); Giuliano et al., Ann. Rev. Biophys. Biomol. Struct. 24:405-434 (1995); and Giuliano et al., Trends in Biotech. 16:135-140 (1998), all references incorporated by reference herein in their entirety.

In this embodiment of the method, the patterned stem cell arrays and patterned differentiated cell arrays are used to analyze changes in the distribution, environment or activity of the luminescent reporter molecules on or in the cells or within or between subcellular compartments of the cells in response to a test compound. The cells are imaged and/or scanned using a cell screening system comprising an optical system with a stage adapted for holding a substrate containing cells, a detection device that is capable of creating a digital image, a means for directing fluorescence or luminescence emitted from the cells to the detection device, and a computer for receiving and processing data from the detection device. A preferred embodiment of such a device is disclosed in U.S. Patent No. 5,989,835; and pending U.S. Patent Application No. 09/031,271 (filed February 27, 1998), both references incorporated by reference herein in

their entirety. Utilizing the cell screening system, luminescent signals from the reporter molecules are converted into digital data; and the digital data is used to determine changes in the distribution, environment or activity of the reporter molecules in response to the test agent.

Such digital data can be used to report the effect of a test compound on distribution of the reporter molecule between: cytoplasm-nucleus, cell membrane-cytoplasm, endoplasmic reticulum-Golgi apparatus, as well as to report on apoptosis; receptor internalization; transcription factor activation; protein kinase activation; protease activity; organelle structure, distribution, and function; macromolecule distribution; gene expression; microtubule cytoskeletal structure; actin cytoskeletal structure; nuclear shape; nuclear area; nuclear size; nuclear perimeter; mitochondrial potential; cell shape; cell motility; cell size; and cell perimeter. (For example, see U.S. Patent No. 5,989,835; pending U.S. Patent Application Nos. 09/031,271 (filed February 27, 1998); 09/352,171 (filed July 12, 1999); 09/293,209 (filed April 16, 1999); 09/398,965 (filed September 17, 1999); 09/430,656 (filed October 29, 1999); and 09/513,783 (filed February 24, 2000).

The use of a fluid delivery system in the method, including but not limited to that disclosed above, or the use of automated precision instruments such as microspotters, permits the delivery of specific cell binding locations with a differentiating agent of choice.

Examples

Organotypic Differentiation Model Systems

Glial differentiation: Cells taken from a highly aggressive human glioblastoma tumor have been shown to grow indefinitely in culture and to exhibit altered morphological and growth characteristics in the presence of a differentiation agent. These cells, named SNB-19 (Welch et al., 1995), are engineered to express a luminescent reporter molecule (see below) and patterned onto cell substrates, either by themselves or in combination with neuronal stem cells. To induce differentiation, a mixture of 1 mM dibutyryl-cAMP and 1 mM isobutylmethyl xanthine (a phosphodiesterase inhibitor) is added to the culture, and the cells are allowed to incubate for 12-

24 hours. These agents induce the cells to elaborate multiple processes that often interact with other glia in the same culture (Welch et al., 1995), as well as cause the cells to stop dividing.

The glial stem cells are transfected to express a green fluorescent protein (GFP)-glial fibrillary acidic protein (GFAP). chimera. GFAP is a component of the intermediate filament cytoskeleton, and is a major cytoskeletal protein found in glial stem cells and differentiated glia.

Neuronal differentiation: Several neuronal stem cell lines exist that can be used in the instant invention. NT2 cells from a human teratocarcinoma cell line (Pleasure et al., 1992) are unique in that they can be induced to differentiate into stable, post-mitotic human neurons, and they have been shown to be a vehicle for the expression of diverse gene products. (Pleasure et al., 1992) NT2 cells are engineered to express a blue fluorescent protein (BFP)-β-tubulin chimera. β-tubulin is a major component of the cytoskeleton. The cells are then patterned onto cell substrates, either by themselves or in combination with glial stem cells. To induce differentiation, NT2 cells initially enter a program of differentiation that begins with a two week treatment of retinoic acid (10^{-5} M), mitotic inhibitors, and a specialized extracellular matrix. The partially differentiated cells are transferred to the substrates where they undergo the final stages of differentiation by elaborating processes that form axons and dendrites. The cells become post-mitotic, but retain the ability to express functional proteins, such as the luminescent protein reporter molecule.

Mixed glial-neuronal differentiation: NT2 stem cells in the final stages of differentiation are added with SNB-19 cells to the same substrate. After both cell types attach, the co-cultures are treated with 1 mM dibutyryl-cAMP and 1 mM isobutylmethyl xanthine. The two cell types are allowed to interact as they differentiate. Because the NT2 cells are committed to differentiation, the dibutyryl-cAMP has little to no effect on neuronal cell differentiation, and may even enhance it, since cAMP is known to induce the differentiation of several cell types.

Adipose and skeletal muscle tissue from a common stem cell: The mouse C2C12 cell line is pluripotent and has been shown capable of differentiating into skeletal muscle (Cuenda and Cohen, 1999), adipocytes (Teboul et al., 1995), and osteoblasts (Nishimura et al., 1998). The

C2C12 cells are first engineered to express a luminescent reporter molecule of carbohydrate metabolism (see below). The cells are patterned onto substrates where the growth medium contains <1% calf serum. This large decrease in serum concentration (10% originally) induces the C2C12 cells, over a period of 24-48 hours, to stop dividing, fuse into elongated, multinucleated cells, and form contractile myotubes. To induce adipocyte differentiation, the cells are treated with a mix of 5 μM thiazolidinedione and 100 μM fatty acid (Teboul et al., 1995). Differentiation occurs after 24-48 hours and is accompanied by the slowing of cell growth and the uptake of fatty acids by the cells and their incorporation into lipid droplets.

The C2C12 cells are transfected with a reporter molecule comprising 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2), which plays a key role in balancing cellular energy utilization and storage. The activity of this bifunctional enzyme can act to switch a cell between carbohydrate oxidation (energy yielding) and carbohydrate synthesis (energy requiring). The phosphorylation state of PFK-2 dictates whether the enzyme will stimulate cellular carbohydrate breakdown or synthesis. (Kurland and Pilkis, Protein Science 4:1023-1037 (1995). The amino acid sequence containing the PFK-2 phosphorylation site is inserted into a specific site within the coding sequence for GFP that tolerates insertions (Baird et al., Proc. Natl. Acad. Sci., 96:11241-11246 (1999)), wherein the fluorescence properties of GFP are altered upon phosphorylation of PFK-2.

Nerve and muscle tissue interaction: Cellular components from the other model systems are combined to model tissue-tissue interactions. Neuronal NT2 cells are combined with C2C12 cells to allow their interaction during differentiation, much like tissues interact develop during normal development. Three scenarios are tested:

1. NT2 and C2C12 cells are arrayed together and allowed to differentiate together;
2. NT2 cells are arrayed on the substrate and differentiated, followed by addition and differentiation of C2C12 cells;
3. C2C12 muscle stem cells are arrayed on the substrate and differentiated, followed by addition and differentiation of neuronal NT2 stems.

Other Examples

Neural crest stem cells can be treated with poly-D-lysine and fibronectin to produce peripheral neurons and glia, as disclosed in U.S. Patent No. 6,033,906. The same stem cells when treated with fibronectin only produce glial cells but not neurons.

Mesenchymal stem cells treated with 100 nM dexamethasone + 10 mM β-glycerophosphate are induced to undergo osteogenesis. (U.S. Patent No. 5,942,225) The same stem cells treated with 5 ng/ml BMP are induced to undergo chondrogenesis; if treated with 5-azacytidine, they are induced to undergo myogenesis. Finally, the same stem cells treated with 10 u/ml IL-1α differentiate into stromal cells.

U.S. Patent No. 5,827,740 teaches treating mesenchymal stem cells with glucocorticoids and phosphodiesterase inhibitor to induce adipogenesis.

Example 6. Fluorescent Biosensor Toxin Characterization

As used herein, "toxin" refers to any organism, macromolecule, or organic or inorganic molecule or ion that alters normal physiological processes found within a cell, or any organism, macromolecule, or organic or inorganic molecule or ion that alters the physiological response to modulators of known physiological processes. Thus, a toxin can mimic a normal cell stimulus, or can alter a response to a normal cell stimulus.

Living cells are the targets of toxic agents that can comprise organisms, macromolecules, or organic or inorganic molecules. A cell-based approach to toxin detection, classification, and identification would exploit the sensitive and specific molecular detection and amplification system developed by cells to sense minute changes in their external milieu. By combining the evolved sensing capability of cells with the luminescent reporter molecules and assays described herein, intracellular molecular and chemical events caused by toxic agents can be converted into detectable spatial and temporal luminescent signals.

When a toxin interacts with a cell, whether it is at the cell surface or within a specific intracellular compartment, the toxin invariably undermines one or more components of the molecular pathways active within the cell. Because the cell is comprised of complex networks of interconnected molecular pathways, the effects of a toxin will likely be transmitted throughout many cellular pathways. Therefore, our strategy is to use molecular markers within key

pathways likely to be affected by toxins, including but not limited to cell stress pathways, metabolic pathways, signaling pathways, and growth and division pathways.

We have developed and characterized three classes of cell based luminescent reporter molecules to serve as reporters of toxic threat agents. These 3 classes are as follows:

(1) *Detectors*: general cell stress detection of a toxin;

(2) *Classifiers*: perturbation of key molecular pathway(s) for detection and classification of a toxin; and

(3) *Identifiers*: activity mediated detection and identification of a toxin or a group of toxins.

Thus, in another aspect of the present invention, living cells are used as biosensors to interrogate the environment for the presence of toxic agents. In one embodiment of this aspect, an automated method for cell based toxin characterization is disclosed that comprises providing an array of locations containing cells to be treated with a test substance, wherein the cells possess at least a first luminescent reporter molecule comprising a detector and a second luminescent reporter molecule selected from the group consisting of a classifier or an identifier; contacting the cells with the test substance either before or after possession of the first and second luminescent reporter molecules by the cells; imaging or scanning multiple cells in each of the locations containing multiple cells to obtain luminescent signals from the detector; converting the luminescent signals from the detector into digital data to automatically measure changes in the localization, distribution, or activity of the detector on or in the cell, which indicates the presence of a toxin in the test substance; selectively imaging or scanning the locations containing cells that were contacted with test sample indicated to have a toxin in it to obtain luminescent signals from the second reporter molecule; converting the luminescent signals from the second luminescent reporter molecule into digital data to automatically measure changes in the localization, distribution, or activity of the classifier or identifier on or in the cell, wherein a change in the localization, distribution, structure or activity of the classifier identifies a cell pathway that is perturbed by the toxin present in the test substance, or wherein a change in the localization, distribution, structure or activity of the identifier identifies the specific toxin that is present in the test substance. In a preferred embodiment, the cells possess at least a detector, a classifier, and an identifier. In a further preferred embodiment, the digital data derived from the

classifier is used to determine which identifier(s) to employ for identifying the specific toxin or group of toxins.

As used herein, the phrase "the cells possess one or more luminescent reporter molecules" means that the luminescent reporter molecule may be expressed as a luminescent reporter molecule by the cells, added to the cells as a luminescent reporter molecule, or luminescently labeled by contacting the cell with a luminescently labeled molecule that binds to the reporter molecule, such as a dye or antibody, that binds to the reporter molecule. The luminescent reporter molecule can be expressed or added to the cell either before or after treatment with the test substance.

The luminescent reporters comprising detectors, classifiers, and identifiers may also be distributed separately into single or multiple cell types. For example, one cell type may contain a toxin detector, which, when activated by toxic activity, implies to the user that the same toxin sample should be screened with reporters of the classifier or identifier type in yet another population of cells identical to or different from the cells containing the toxin detector.

The detector, classifier, and identifier can comprise the same reporter molecule, or they can comprise different reporters.

Screening for changes in the localization, distribution, structure or activity of the detectors, classifiers, and/or identifiers can be carried out in either a high throughput or a high content mode. In general, a high-content assay can be converted to a high-throughput assay if the spatial information rendered by the high-content assay can be recoded in such a way as to no longer require optical spatial resolution on the cellular or subcellular levels. For example, a high-content assay for microtubule reorganization can be carried out by optically resolving luminescently labeled cellular microtubules and measuring their morphology (*e.g.*, bundled vs. non-bundled or normal). A high-throughput version of a microtubule reorganization assay would involve only a measurement of total microtubule polymer mass after cellular extraction with a detergent. That is, destabilized microtubules, being more easily extracted, would result in a lower total microtubule mass luminescence signal than unperturbed or drug-stabilized luminescently labeled microtubules in another treated cell population. The luminescent signal emanating from a domain containing one or more cells will therefore be proportional to the total microtubule mass remaining in the cells after toxin treatment and detergent extraction.

The methods for detecting, classifying, and identifying toxins can utilize the same screening methods described throughout the instant application, including but not limited to detecting changes in cytoplasm to nucleus translocation, nucleus or nucleolus to cytoplasm translocation, receptor internalization, mitochondrial membrane potential, signal intensity, the spectral response of the reporter molecule, phosphorylation, intracellular free ion concentration, cell size, cell shape, cytoskeleton organization, metabolic processes, cell motility, cell substrate attachment, cell cycle events, and organellar structure and function.

In all of these embodiments, the methods can be operated in both toxin-mimetic and toxin-inhibitory modes.

Such techniques to assess the presence of toxins are useful for methods including, but not limited to, monitoring the presence of environmental toxins in test samples and for toxins utilized in chemical and biological weapons; and for detecting the presence and characteristics of toxins during environmental remediation, drug discovery, clinical applications, and during the normal development and manufacturing process by virtually any type of industry, including but not limited to agriculture, food processing, automobile, electronic, textile, medical device, and petroleum industries.

We have developed and characterized examples of luminescent cell-based reporters, distributed across the 3 sensor classes. The methods disclosed herein can be utilized in conjunction with computer databases, and data management, mining, retrieval, and display methods to extract meaningful patterns from the enormous data set generated by each individual reporter or a combinatorial of reporters in response to toxic agents. Such databases and bioinformatics methods include, but are not limited to, those disclosed in U.S. Patent Application Nos. 09/437,976, filed November 10, 1999; 60/145,770 filed July 27, 1999 and U.S. Patent Application Serial No. to be assigned, filed February 19, 2000. (98,068-C)

Any cell type can be used to carry out this aspect of the invention, including prokaryotes such as bacteria and archaebacteria, and eukaryotes, such as single celled fungi (for example, yeast), molds (for example, Dictyostelium), and protozoa (for example, Euglena). Higher eukaryotes, including, but not limited to, avian, amphibian, insect, and mammalian cells can also be used.

Table 3. Examples of Biosensors

#	Name	Class	Cell Types	Response to model toxins Positive	Negative
1	Mitochondrial Potential [Donnan Equilibrium Dye]	D	• LLCPK (pig epithelia) • Rat primary hepatocytes	Valinomycin (10 nM-100 μM) FCCP (10 nM-100 μM)	Oligomycin (10 nM)
2	Heat Shock Protein (Hsp 27, Hsp 70) GFP-chimera	D	• HeLa • 3T3	Cadmium (10mM)	TNF-α (100ng/ml)
3	Tubulin-cytoskeleton [β-tubulin-GFP chimera]	C	• BHK • HeLa • LLCPK	Paclitaxel (10 nM-20μM) Curacin-A (5 nM-10μM) Nocadazole (7 nM-12μM) Colchicine (5 nM-10μM) Vinblastine (5 nM-10μM)	Staurosporine (1 nM-1 μM)
4	pp38 MAPK- stress signaling [antibody and GFP-chimera]	C	• 3T3 • LLCPK	Anisomycin (100 μM) Cadmium (10 μM)	TNF-α (100 ng/ml)
5	NF-κB- stress signaling [antibody and GFP-chimera]	C	• HeLa • 3T3 • BHK • SNB19 • HepG2 • LLCPK	TNF-α (100ng/ml-0.38pg/ml) IL-1 (4ng/ml-.095pg/ml) Nisin (2-1000 μg/ml) Streptolysin (10 μg/ml) Anisomycin (100 μM)	Anisomycin (10 nM-10 μM) Cadmium (1-10 μM) Penitrem A (10 μM) Valinomycin (1 μM)
6	IκB [complement to NF-κB]	C	In many cell types		
7	Tetanus Toxin [Protease activity-based sensor]	I	In many cell types		
8	Anthrax LF [Protease activity-based sensor]	I	In many cell types		

Sensor Class: D= Detector of toxins; C= Classifier of toxins; I= Identifier of toxin or group of toxins

The model toxins can generally be purchased from Sigma Chemical Company (St. Louis, MO)

Examples of Detectors: This class of sensors provides a first line signal that indicates the presence of a toxic agent. This class of sensors provides detection of general cellular stress that requires resolution limited only to the domain over which the measurement is being made, and they are amenable to high content screens as well. Thus, either high throughput or high content screening modes may be used, including but not limited to translocation of heat shock factors from the cytoplasm to the nucleus, and changes in mitochondrial membrane potential, intracellular free ion concentration detection (for example, Ca^{2+}; H^{+}), general metabolic status, cell cycle timing events, and organellar structure and function.

1. Mitochondrial Potential

A key to maintenance of cellular homeostasis is a constant ATP energy charge. The cycling of ATP and its metabolites ADP, AMP, inorganic phosphate, and solution-phase protons is continuously adjusted to meet the catabolic and anabolic needs of the cell. Mitochondria are primarily responsible for maintaining a constant energy charge throughout the entire cell. To produce ATP from its constituents, mitochondria must maintain a constant membrane potential within the organelle itself. Therefore, measurement of this electrical potential with specific luminescent probes provides a sensitive and rapid readout of cellular stress.

We have utilized a positively charged cyanine dye, JC-1 (Molecular Probes, Eugene, OR), which diffuses into the cell and readily partitions into the mitochondrial membrane, for measurement of mitochondrial potential. The photophysics of JC-1 are such that when the probe partitions into the mitochondrial membrane and it experiences an electrical potential >140 mV, the probe aggregates and its spectral response is shifted to the red. At membrane potential values <140 mV, JC-1 is primarily monomeric and its spectral response is shifted toward the blue. Therefore, the ratio of two emission wavelengths (645 nm and 530 nm) of JC-1 partitioned into mitochondria provides a sensitive and continuous measure of mitochondrial membrane potential.

We have been making live cell measurements in a high throughput mode as the basis of a generalized indicator of toxic stress. The goal of our initial experiments was to determine the ratio of J-aggregates of JC-1 dye to its monomeric form both before and after toxic stress.

Procedure

1. Cells were plated and cultured up to overnight.
2. Cells were stained with JC-1 (10 μg/ml) for 30 minutes at 37° C in a CO_2 incubator.
3. Cells were then washed quickly with HBSS at 37°C (2 times, 150 μl/well), the toxins were added if required, and the entire plate was scanned in a plate reader. The JC-1 monomer was measured optimally with a 485 nm excitation/530 nm emission wavelength filter set, and the aggregates were best measured with a 590 nm excitation/645 nm emission wavelength set.

Results

The mitochondrial potential within several types of living cells, and the effects of toxins on the potential were measured using the fluorescence ratio Em 645 (590)/ Em 530 (485) (excitation wavelengths in parentheses). For example, we measured the effect of 10 μM valinomycin on the mitochondrial potential within LLCPK cells (pig epithelia). Within seconds of treatment, the toxin induced a more rapid and higher magnitude decrease (an approximately 50% reduction) in mitochondrial potential than that found in untreated cells. Hepatocytes were also determined to be sensitive to valinomycin, and the changes in mitochondrial potential were nearly complete within seconds to minutes after addition of various concentrations of the toxin.

These results are consistent with mitochondrial potential being a model intracellular detector of cell stress. Because these measurements require no spatial resolution within individual cells, mitochondrial potential measurements can be made rapidly on an entire cell array (e.g. high throughput). This means, for example, that complex arrays of many cell types can be probed simultaneously and continuously as a generalized toxic response. Such an indicator can provide a first line signal to indicate that a general toxic stress is present in a sample. Further assays can then be conducted to more specifically identify the toxin using cells classifier or identifier type reporter molecules.

2. Heat Shock Proteins

Most mammalian cells will respond to a variety of environmental stimuli with the induction of a family of proteins called stress proteins. Anoxia, amino acid analogues, sulfhydryl-reacting reagents, transition metal ions, decouplers of oxidative phosphorylation, viral

infections, ethanol, antibiotics, ionophores, non-steroidal antiinflammatory drugs, thermal stress and metal chelators are all inducers of cell stress protein synthesis, function, or both. Upon induction, cell stress proteins play a role in folding and unfolding proteins, stabilizing proteins in abnormal configurations, and repairing DNA damage.

There is evidence that at least four heat shock proteins translocate from the cytoplasm to the nucleus upon stress activation of the cell. These proteins include the heat shock proteins HSP27 and HSP70, the heat shock cognate HSC70, and the heat shock transcription factor HSF1. Therefore, measurement of cytoplasm to nuclear translocation of these proteins (and other stress proteins that translocate from the cytoplasm to the nucleus upon a cell stress) will provide a rapid readout of cellular stress.

We have tested the response of an HSP27-GFP biosensor in two cell lines (BHK and HeLa) using a library of heavy metal chemical compounds as biological toxin stimulants to stress the cells. Briefly, cells expressing the HSP27-GFP biosensor are plated into 96-well microplates, and allowed to attach. The cells are then treated with a panel of cell stress-inducing compounds. Exclusively cytoplasmic localization of the fusion protein was found in unstimulated cells.

Other similar heat shock protein biosensors (HSP-70, HSC70, and HSF1 fused to GFP) can also be used as detectors.

Examples of Classifiers:

This class of sensors detects the presence of, and further classifies toxins by identifying the cellular pathway(s) perturbed by the toxin. As such, this suite of sensors can detect and/or classify toxins into broad categories, including but not limited to "toxins affecting signal transduction," "toxins affecting the cytoskeleton," and "toxins affecting protein synthesis". Either high throughput or high content screening modes may be used. Classifiers can comprise compounds including but not limited to tubulin, microtubule-associated proteins, actin, actin-binding proteins including but not limited to vinculin, α-actinin, actin depolymerizing factor/cofilin, profilin, and myosin; NF-κB, IκB, GTP-binding proteins including but not limited to rac, rho, and cdc42, and stress-activated protein kinases including but not limited to p38 mitogen-activated protein kinase.

1. *Tubulin-cytoskeleton*

The cell cytoskeleton plays a major role in cellular functions and processes, such as endo- and exocytosis, vesicle transport, and mitosis. Cytoskeleton-affecting toxins, of proteinaceous and non-proteinaceous form, such as C2 toxin, and several classes of enterotoxins, act either directly on the cytoskeleton, or indirectly via regulatory components controlling the organization of the cytoskeleton. Therefore, measurement of structural changes in the cytoskeleton can provide classification of the toxin into a class of cytoskeleton-affecting toxins. This assay can be conducted in a high content mode, as described previously, or in a high throughput mode. For high throughput as discussed previously.

Such measurements will be valuable for identification of toxins including, but not limited to anti-microtubule agents, agents that generally affect cell cycle progression and cell proliferation, intracellular signal transduction, and metabolic processes.

For microtubule disruption assays, LLCPK cells stably transfected with a tubulin-GFP biosensor plasmid were plated on 96 well cell culture dishes at 50-60% confluence and cultured overnight at 37 °C, 5% CO_2. A series of concentrations (10–500 nM) of 5 compounds (paclitaxel, curacin A, nocodazole, vinblastine, and colchicine) in normal culture media were freshly prepared from stock, and were added to cell culture dishes to replace the old culture media. The cells were then observed with the cell screening system described above, at a 12 hour time point.

Our data indicate that the tubulin chimera localizes to and assembles into microtubules throughout the cell. The microtubule arrays in cells expressing the chimera respond as follows to a variety of anti-microtubule compounds:

Drug	Response
Vinblastine	Destabilization
Nocodazole	Destabilization
Paclitaxel	Stabilization
Colchicine	Destabilization
Curacin A	Destabilization

Similar data were obtained using cells expressing the tubulin biosensor that were patterned onto cell arrays (such as those described in U.S. Patent Application Serial No. 08/865,341 filed May 29, 1997, incorporated by reference herein in its entirety) and dosed as above.

2. *NF-κB*

NF-κB is cytoplasmic at basal levels of stimulation, but upon insult translocates to the nucleus where it binds specific DNA response elements and activates transcription of a number of genes. Translocation occurs when IkB is degraded by the proteosome in response to specific phosphorylation and ubiquitination events. IkB normally retains NF-κB in the cytoplasm via direct interaction with the protein, and masking of the NLS sequence of NF-κB. Therefore, although not the initial or defining event of the whole signal cascade, NF-κB translocation to the nucleus can serve as an indicator of cell stress.

NF-κB immunolocalization

For further studies, we characterized endogenous NF-κB activation by immunolocalization in toxin treated cells. The NF-κB antibodies used in this study were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and secondary antibodies are from Molecular Probes (Eugene, OR).

For the 3T3 and SNB19 cell types, we determined the effective concentrations that yield response levels of 50% of the maximum (EC50), expressed in units of mass per volume (ng/ml) and units of molarity. Based on molecular weights of 17 kD for both TNFα and IL-1α, the EC50 levels for these two compounds with 3T3 and SNB19 cell types are given in units of molarity in **Table 3**. Our results demonstrated reproducibility of the relative responses from zero to maximum dose, but from sample to sample there have been occasional shifts in the baseline intensities of the response at zero concentration.

For these experiments, either 10 or 100 TNFα-treated 3T3 or SNB19 cells/well were tested. On the basis of the standard deviations measured for these samples, and by taking t-values for the student's t-test, we have estimated the minimum detectable doses for each case of

cell type, compound, number of cells per well, and for different choices of how many wells are sampled per condition. The latter factor determines the number of degrees of freedom that are provided in the sample of data. Increasing the number of wells from 4 to 16, and increasing the number of cells per well from 10 to 100, improves the minimum detectable doses considerably. For 3T3 cells, which show lower minimum detectable doses than the SNB19 cells, and for the case of 1% false negative and 1% false positive rates, we estimate that 100 cells per well and a sampling of 12 or 16 wells are sufficient to detect a dose approximately equal to the EC50 value of 0.15 ng/ml. If the false positive rate is relaxed to 20%, a concentration of approximately half that value can be detected (0.83 ng/ml). One hundred cells can conveniently be sampled over a cell culture surface area of less than 1 mm^2.

Table 3. EC50 levels for TNFα and IL-1α (based on molecular weights of 17 kD for both)

Compound	Cell Type	EC50 (10^{-12} moles/liter)
TNFα	3T3	8.8
	SNB19	5.9
IL-1α	3T3	0.24
	SNB19	59

3. Phospho-p38 Mitogen Activated Protein Kinase (pp38MAPK)

MAPKs play a role in not only cell growth and division, but as mediators of cellular stress responses. One MAPK, p38, is activated by chemical stress inducers such as hyper-osmolar sorbitol, hydrogen peroxide, arsenite, cadmium ions, anisomycin, sodium salicylate, and LPS. Activation of p38 is also accompanied by its translocation into the nucleus from the cytoplasm.

MAPK p38 lies in a pathway that is a cascade of kinases. Thus, p38 is a substrate of one or more kinases, and it acts to phosphorylate one or more substrates in time and space within the living cell.

The assay we present here measures, as one of its parameters, p38 activation using immunolocalization of the phosphorylated form of p38 in toxin-treated cells. The assay was

developed to be flexible enough to include the simultaneous measurement of other parameters within the same individual cells. Because the signal transduction pathway mediated by the transcription factor NF-κB is also known to be involved in the cell stress response, we included the activation of NF-κB as a second parameter in the same assay.

Our experiments demonstrate an immunofluorescence approach can be used to measure p38 MAPK activation either alone or in combination with NF-κB activation in the same cells. Multiple cell types, model toxins, and antibodies were tested, and significant stimulation of both pathways was measured in a high-content mode. The phospho-p38 antibodies used in this study were purchased from Sigma Chemical Company (St. Louis, MO). We report that at least two cell stress signaling pathways can not only be measured simultaneously, but are differentially responsive to classes of model toxins. **Figure 46** shows the differential response of the p38 MAPK and NF-κB pathways across three model toxins and two different cell types. Note that when added alone, three of the model toxins (IL1α, TNFα and Anisomycin) can be differentiated by the two assays as activators of specific pathways.

IκB chimera

IkB degradation is the key event leading to nuclear translocation of NF-kB and activation of the NFkB-mediated stress response. We have chosen this sensor to complement the NF-kB sensor as a *classifier* in a high-throughput mode: the measurement of loss of signal due to degradation of the IκB-GFP fusion protein requires no spatial resolution within individual cells, and as such we envision IkB degradation measurements being made rapidly on an entire cell substrate.

This biosensor is based on fusion of the first 60 amino acids of IkB to the Fred25 variant of GFP. This region of IkB contains all the regulatory sequences, including phosphorylation sites and ubiquitination sites, necessary to confer proteosome degradation upon the biosensor. Knowing this, stimulation of any pathway that would typically lead to NFkB translocation results in degradation of this biosensor. Monitoring the fluorescence intensity of cells expressing IkB-GFP identifies the degradation process.

Examples of Identifiers:

In our toxin identification strategy, the first two levels of characterization ensure a rapid readout of toxin class without sacrificing the ability to detect many new mutant toxins or dissect several complex mixtures of known toxins. The third level of biosensors are identifiers, which can identify a specific toxin or group of toxins. In one embodiment, an identifier comprises a protease biosensor that responds to the activity of a specific toxin. Other identifiers are produced with reporters/biosensors specific to their activities. These include, but are not limited to post-translational modifications such as phosphorylation or ADP-ribosylation, translocation between cellular organelles or compartments, effects on specific organelles or cellular components (for example, membrane permeabilization, cytoskeleton rearrangement, etc.)

ADP-ribosylating toxins – These toxins include Pseudomonas toxin A, diptheria toxin, botulinum toxin, pertussis toxin, and cholera toxin. For example, C. botulinum C2 toxin induces the ADP-ribosylation of Arg177 in the cytoskeletal protein actin, thus altering its assembly properties. Besides the construction of a classifier assay to measure actin-cytoskeleton regulation, an identifier assay can be constructed to detect the specific actin ADP-ribosylation. Because the ADP-ribosylation induces a conformational change that no longer permits the modified actin to polymerize, this conformational change can be detected intracellularly in several possible ways using luminescent reagents. For example, actin can be luminescently labeled using a fluorescent reagent with an appropriate excited state lifetime that allows for the measurement of the rotational diffusion of the intracellular actin using steady state fluorescence anisotropy. That is, toxin-modified actin will no longer be able to assemble into rigid filaments and will therefore produce only luminescent signals with relatively low anisotropy, which can be readily measured with an imaging system. In another embodiment, actin can be labeled with a polarity-sensitive fluorescent reagent that reports changes in actin-conformation through spectral shifts of the attached reagent. That is, toxin-treatment will induce a conformational change in intracellular actin such that a ratio of two fluorescence wavelengths will provide a measure of actin ADP-ribosylation.

Cytotoxic phospholipases – Several gram-positive bacterial species produce cytotoxic phospholipases. For example, Clostridium perfringens produces a phospholipase C specific for

the cleavage of phosphoinositides. These phosphoinositides (e.g., inositol 1,4,5-trisphosphate) induce the release of calcium ions from intracellular organelles. An assay that can be conducted as either high-content or high-throughput can be constructed to measure the release of calcium ions using fluorescent reagents that have altered spectral properties when complexed with the metal ion. Therefore, a direct consequence of the action of a phospholipase C based toxin can be measured as a change in cellular calcium ion concentration.

Exfoliative toxins – These toxins are produced by several Staphylococcal species and can consist of several serotypes. A specific identifier for these toxins can be constructed by measuring the morphological changes in their target organelle, the desmosome, which occur at the junctions between cells. The exfoliative toxins are known to change the morphology of the desmosomes into two smaller components called hemidesmosomes. In the high-content assay for exfoliative toxins, epithelial cells whose desmosomes are luminescently labeled are subjected to image analysis. An method that detects the morphological change between desmosomes and hemidesmosomes is used to quantify the activity of the toxins on the cells.

Most of these identifiers can be used in high throughput assays requiring no spatial resolution, as well as in high content assays.

Several biological threat agents act as specific proteases, and thus we have focused on the development of fluorescent protein biosensors that report the proteolytic cleavage of specific amino acid sequences found within the target proteins.

A number of such protease biosensors (including FRET biosensors) are disclosed above, such as the caspase biosensors, anthrax, tetanus, Botulinum, and the zinc metalloproteases. FRET is a powerful technique in that small changes in protein conformation, many of which are associated with toxin activity, can not only be measured with high precision in time and space within living cells, but can be measured in a high-throughput mode, as discussed above.

As described above, one of skill in the art will recognize that the protease biosensors of this aspect of the invention can be adapted to report the activity of any protease, by a substitution of the appropriate protease recognition site. These biosensors can be used in high-content or high throughput screens to detect in vivo activation of enzymatic activity by toxins, and to identify specific activity based on cleavage of a known recognition motif. These biosensors can

be used in both live cell and fixed end-point assays, and can be combined with additional measurements to provide a multi-parameter assay.

Anthrax LF

Anthrax is a well-known agent of biological warfare and is an excellent target for development of a biosensor in the *identifier* class. Lethal factor (LF) is one of the protein components that confer toxicity to anthrax, and recently two of its targets within cells were identified. LF is a metalloprotease that specifically cleaves Mek1 and Mek2 proteins, kinases that are part of the MAP-kinase signaling pathway. Green fluorescent protein (GFP) is fused in-frame at the amino terminus of either Mek1 or Mek2 (or both), resulting in a chimeric protein that is retained in the cytoplasm due to the presence of a nuclear export sequence (NES) present in both of the target molecules. Upon cleavage by active lethal factor, GFP is released from the chimera and is free to diffuse into the nucleus. Therefore, measuring the accumulation of GFP in the nucleus provides a direct measure of LF activity on its natural target, the living cell.

Multi-parametric screens

Multiparameter high-content assays for toxin activity within a single population of cells has several advantages over single parameter assays:

1. The reagent and cell-handling overhead is greatly reduced over multiple single parameter assays.
2. The time requirements for sample preparation and analysis are greatly reduced.
3. Reagents can be conserved.
4. Most importantly, the biological analysis and the building of cellular knowledge can be greatly enhanced when multiple toxin activity parameters are measured within single cells.

Presented here is an analysis of multiple physiological targets within multiple single cell types. The results are consistent with toxin identification and characterization. The specific toxins chosen for the demonstration were *Staphylococcus* Enterotoxin B, (SEB), tumor necrosis factor alpha (TNFα), and botulinum toxin.

To complete the analysis, the following strategy was pursued:

1. Choose intracellular targets known to be modulated by SEB and botulinum toxins and substitute analogs that can be used to verify the assay. SEB is known to activate p38 MAPK in some cell types. The botulinum toxin C3 exoenzyme is known to affect the organization of the actin-cytoskeleton through its ADP-ribosylation activity. One well-characterized effect of TNFα is the stimulation of NF-κB transcriptional activity. Furthermore, it is also known that there is cross talk between the pathways activated by all three of the above toxins. Therefore, a combinatorial approach was used to measure the activation of cytoskeletal, MAPK, and NF-κB pathways within the same single cells.

2. Choose toxin analogs known to stimulate the above pathways. To simulate the activity of the above toxins, the following compounds and ions were proposed for use as toxin analogs:
   i) Anisomycin [SEB analog] – This drug is known to activate the p38 MAPK pathway through increased phosphorylation of p38. Measurement is done using antibodies against the phosphorylated (activated) form of p38 and the cytoplasm to nuclear translocation application.
   ii) TNFα [TNFα] – This cytokine is known to activate the NF-κB pathway and is measurable as the cytoplasm to nuclear translocation of the transcription factor.
   iii) Latrunculin [botulinum C3 exoenzyme analog] – This drug is known to affect the equilibrium between globular and filamentous actin in living cells. This shift in equilibrium is measurable as a change in the intensity of fluorescent phalloidin labeling of single cells.

Cell plating and drug treatment. Swiss 3T3 cells were plated at 15,000 cells per well in a 96-well microplate 8 hours before drug treatment. Columns consisting of 8 wells within the plate were treated with the following concentration ranges of drugs: TNFα, 0.781-10 ng/ml;

anisomycin, 7.81-1000 nM; and latrunculin, 2.5-320 nM. The cells were treated with the drugs for 30 minutes at 37° C in a humidified 5% CO_2 atmosphere.

Cell labeling. After drug incubation, the medium was removed from the wells and replaced with a solution (175 μl/well) containing 4% formaldehyde and 10 μg/ml Hoechst 33342 in Hank's Balanced Salt Solution (HBSS). The cells were incubated in this solution for 20 minutes at room temperature. The wells were rinsed once with HBSS (200 μl/well), and then treated for 5 minutes at room temperature with a solution (200 μl/well) containing 0.5% (v/v) Triton X-100 in HBSS. After this detergent extraction, the cells were rinsed as above and incubated with a solution (50 μl/well) containing a 1:500 dilution of a mouse monoclonal antibody to phosphorylated p38 MAPK, a 1:200 dilution of a rabbit polyclonal antibody to NF-κB, and a 1:400 dilution of Alexa 488 labeled phalloidin for 1 hour at room temperature. After rinsing as above, the cells were incubated one last time with a solution (50 μl/well) containing a 1:150 dilution of Alexa 568-labeled polyclonal anti-mouse antibody solution and a 1:150 dilution of a Cy5-labeled polyclonal anti-rabbit antibody solution for 1 hour at room temperature. The cells were rinsed as above, and stored at 4° C in a sealed plate containing HBSS (200 μl/well).

High-content analysis of labeled cells. The 96-well microplate containing the multiply labeled cells was scanned with a cell screening system, including but not limited to those disclosed in U.S. Patent No. 5,989,835 and in U.S. Patent Application Serial No. 09/031,271 filed February 27, 1998, both incorporated by reference herein in their entirety. Cytoplasm to nuclear ratios were made for p38 MAPK, NF-κB, and actin within the same population of cells.

Results and Discussion

Table 4 shows the results of the multiparameter measurement of three intracellular activities as a function of the concentration of three different toxins. NF-κB translocation is shown to be sensitive to TNFα concentration, but p38 MAPK and actin-cytoskeleton assembly state in the same cells are relatively insensitive. p38 MAPK activation is more sensitive than NF-κB translocation to increasing anisomycin concentration. Here again, actin-cytoskeletal

assembly state is relatively unaffected by this drug. High concentrations of latrunculin induce a reorganization of the actin-cytoskeleton, but have relatively little effect on p38 MAPK activation or NF-κB translocation in the same cells.

Table 4. Maximal Cellular Response Relative to Control*

Analog	NF-κB	p38 MAPK	Actin
TNFα	1.8 (> 25 nM)	1.2 (> 200 nM)	1.0
Anisomycin	1.0	1.4 (> 200 nM)	1.0
Latrunculin	1.0	1.0	1.2 (>150 nM)

***A maximal cellular response of 1.0 indicates no significant difference over control values.**

The analysis of cellular data using multiparameter high-content assays demonstrated here provides new insights into the mechanisms of both known and unknown toxin activity. For example, TNFα acts as a toxin through a molecular pathway that uses NF-κB translocation to activate specific cellular genes, while anisomycin action is consistent with the activation of both p38 MAPK and NF-κB pathways. The further application of this multiparameter approach where comparisons are made at the single cell level also greatly increases the resolution to which toxin action can be dissected and therefore improves the precision and sensitivity of toxin detection.

Example 12 Matrix for toxin detection and organ localization

In another aspect, the present invention provides a cassette for cell screening, comprising a substrate having a surface; and a fluid delivery system mated with the substrate, wherein the fluid delivery system comprises (1) a matrix of openings or depressions, wherein each region of the substrate enclosed by the opening or depression in the matrix comprises an individually addressable domain; and (2) microfluidic channels that supply fluid to the domains. The architecture of the fluidic channels is governed by the laws of fluid flow in microchannels alone.

In this aspect, the microfluidic device consists of a matrix of either openings or depressions with dimensions ranging from 1 μm to 10,000 μm per side, or 1 μm to 10,000 μm in

diameter with depths ranging from 10 μm to 10,000 μm. Such a matrix can comprise any desired number of rows and columns.

In one embodiment, a 3 X 3 matrix comprises openings, which when bonded to the substrate permits modification of the substrate surface with adhesion chemistry and cells. Subsequently, the openings are closed, and the closed cassette is used for screening by pumping fluids and reagents into the assay domains via the microfluidic channels. The microfluidic device can be bonded to the substrate via such non-limiting means as anodic bonding, electrofusion, thermal bonding, adhesive bonding, and pressure bonding. Each region on the substrate enclosed by an opening or depression now constitutes a "domain" on the substrate.

In another embodiment of this aspect of the invention, the substrate comprises cell binding locations separated by cell repulsive regions, and cells are arrayed on the cell binding locations. In a preferred embodiment, each domain on the substrate comprises a single cell type. The nine domains on the substrate are thus preferably arrayed with between one and nine different cell types, with each cell type bearing one or more biosensors reporting on one or more intracellular pathways. In further preferred embodiments, at least three different cell types are arrayed on the substrate; and the different cell types arrayed on the substrate are specific for different tissue types, including but not limited to cells specific for connective tissue, neuronal tissue, and the immune system.

As such, each of the nine domains has a single cell type arrayed on cell binding locations. Each of the nine domains are individually addressable by a fluid of choice using the microfluidic device. If the fluid delivery device comprises a matrix of openings, the top of the device is sealed from the environment with a glass or plastic film, or an air permeable and water impermeable membrane such as BREATHEASY™ (Sigma Chemical Co.), by means such as, but not limited to anodic bonding, anodic bonding, electrofusion, thermal bonding, adhesive bonding, and pressure bonding. The semi-permeable membrane enables oxygen and carbon dioxide diffusion for long-term viability and functionality of the cells.

Cell adhesion promoters and inhibitors that can be used include, but are not limited to, those discussed throughout the application. In a most preferred embodiment, the cell adhesion promoter comprises a silane, and the cell repulsive moiety comprises tresyl-activated PEG. The surface modifications can be achieved by either transferring cell adhesive molecules onto the

substrate in select regions, or by coating select regions of the substrate with cell repulsive molecules via any method, including but not limited to those described above. The substrate so modified is arrayed with cells in each of the regions enclosed by the square openings or depressions. Alternatively, the substrate can be modified with cell adhesive and cell selective chemistries after bonding to the microfluidic device. Where the fluid delivery system comprises a matrix of openings, the chemistries and cells can be arrayed on the substrate either via the microfluidic channels, or via the openings in the fluid delivery system, after which the wells are closed with a glass film, plastic film, or air permeable and water impermeable membrane as described above. Where the fluid delivery system comprises a matrix of depressions, the chemistries and cells can be delivered via the microfluidic channels.

In another aspect, the invention provides a method for cell screening, comprising providing the cassette for cell screening described in this example, providing an optical system to obtain images of the cells; contacting the domains with a test compound; and obtaining images of the cells to determine an effect of the test compound on the cells. Such screening can be conducted in either a high content mode, a high throughput mode or a combination of the two. The high throughput mode permits optical detection of signals arising from all domains on the substrate in one pass, while the high content mode permits sub-cellular resolution and detection of signals arising from each individual cell within each domain.

In a preferred embodiment, the method detects the pathway and organ localization of a toxin, wherein the substrate comprises multiple cell types each with one or more biosensors that are affected by a toxin; contacting the domains with a test sample potentially comprising a toxin, and obtaining images of the cells to determine an effect of the toxin on the different cell types, wherein an effect of the toxin on the cell types indicates the toxin pathway and organ localization. As used herein, "organ localization" means that a particular toxin has an effect on cells from a particular organ, and thus its toxicity may be mediated, at least in part, through that organ. The term does not imply that cells from other organs are not affected by the toxin.

In a further preferred embodiment, the biosensors expressed by the cells are selected from the group consisting of detectors, classifiers, and identifiers, as described above.

In a further aspect, the invention further provides an automated method for cell based toxin detection and organ localization comprising

-providing an array of locations containing cells to be treated with a test substance, wherein the array comprises at least a first cell type and a second cell type, and wherein the first cell type and the second cell type are not contained on the same location in the array; wherein the first and second cell types are derived from different organ types; wherein each of the cell types comprises at least one luminescent reporter molecule; wherein the localization, distribution, structure, or activity of the at least one luminescent reporter molecule is altered by a toxin to be detected;

-contacting the at least first cell type and second cell type with the test substance either before or after possession of the at least one luminescent reporter molecules by the first cell type and the second cell type;

-imaging or scanning multiple cells in each of the locations containing the first cell type or the second cell type to obtain luminescent signals from the luminescent reporter molecule in the first cell type and the second cell type;

-converting the luminescent signals into digital data;

-utilizing the digital data to automatically measure the localization, distribution, structure or activity of the at least one luminescent reporter molecule on or in the first cell type and the second cell type, wherein a change in the localization, distribution, structure or activity of the luminescent reporter molecule indicates the presence of a toxin and an organ localization of the toxin.

In a preferred embodiment, the array further comprise a third cell type, wherein the first, second, and third cell types are not contained on the same location in the array; and wherein the first, second, and third cell types are derived from different organ types.

In a further preferred embodiment, the cell types further comprise at least a second luminescent reporter molecule; wherein the localization, distribution, structure, or activity of the second luminescent reporter molecule is altered by a toxin to be detected.

For developing a 3 x 3 matrix of assays and toxin analogs for multi-parametric toxin identification and organ localization, several factors were considered, *including* the cellular events or pathways that are affected by toxic agents, the critical biochemical targets that undergo a measurable change *in response to a toxin*, and whether *such* changes in the critical biochemical target are measurable. The following are representative examples of such assays, toxins, and

toxin analogs that can be used in accordance with the present invention. One of skill in the art will recognize that many other such assays, toxins, and toxin analogs can be used according to the teachings of the present invention, all of which are encompassed by the present invention.

The shape of a cell is determined in large part by its actin cytoskeleton. Upon loss of F-actin, cells undergo profound shape changes. Botulinum C3 toxin is a member of a class of toxins that target the Ras signaling pathway. Botulinum C3 toxin inactivates Rho, leading to actin depolymerization, thus causing a detectable change in cell morphology, particularly a decrease in cell area. Similarly to Botulinum C3, cytochalasin D binds to actin and prevents polymerization, leading to loss of cytoskeletal organization. Thus, cytochalasin D can be used as a Botulinum C3 analog to demonstrate the ability of the toxin assay to classify a toxin present in the sample as affecting cell morphology in a manner similar to Botulinum C3. F-actin can be labeled with fluorescent phalloidin to reveal the cytoskeletal structure, and changes in such structure can be measured in response to a test sample containing cytochalasin D. Thus, the present assay serves to classify the toxin as one that perturbs the cell's actin filament structure.

Botulinum C3 also induces the expression of cell stress proteins, and thus would be detected as a toxin by a cell stress protein biosensor, such as those described above. Botulinum C3 also affects membrane trafficking.

The p38 MAP kinase, (pp38MAPK, discussed above), is phosphorylated and translocates to the nucleus upon activation of the MAP kinase pathway. Antibodies specific for phosphorylated p38 (the target of pp38MAPK) report its location in the cell. pp38MAPK is also a target of Staphylococcal enterotoxin B (SEB) and T2 Toxin. SEB alters the function of immune cells and stimulates p38 activation and translocation in some T-cell lines. Certain tricothecene mycotoxins (e.g. T2 *toxins such as T2 triol*) inhibit protein synthesis and induce p38 activity. Anisomycin also inhibits protein synthesis and induces p38 translocation to the nucleus as discussed above, and thus can be used as an analog of SEB and T2 toxin, to demonstrate the ability of the toxin assay to classify a toxin present in the sample as affecting p38 activation and/or localization in a manner similar to SEB and/or T2 toxins. Furthermore, by utilizing cells specific for the immune system, the assay provides information on toxin localization in the body.

SEB also induces the expression of cell stress proteins, and thus could be detected in a test sample as a toxin by a cell stress protein biosensor, such as those described above. SEB also

affects microtubule structure, and its presence in a test sample would lead to a classification of the test sample as containing a toxin that perturbs the cell microtubule structure.

Cells respond to environmental agents of stress (as discussed above) through specific signaling cascades. As discussed above, NFkB is a transcription factor that activates production of proteins as part of the cellular defense response to general cell stress. NFkB resides in the cytoplasm but translocates to the nucleus upon release from a complex with IκB. Antibody staining reveals the subcellular localization of NFkB. TNF-α is a strong inducer of NF-κB translocation to the nucleus, and thus can be used as an analog of toxins that induce cell stress, including but not limited to cholera toxin.

Table 5. Matrix 1: Dissection of pathway and organ localization of 1 toxin per cassette

	Toxin 1	Toxin 1	Toxin 1
	1	2	3
A	SD3T3 (HSP)	SD3T3 (NF-kB)	SD3T3 (β-tubulin)
B	SNB19 (HSP)	SNB19 (NF-kB)	SNB19 (β-tubulin)
C	RAW (HSP)	RAW (NF-kB)	RAW (β-tubulin)

This example permits addressing the effect of a single toxin on connective tissue, neuronal tissue, and immune specific cells as measured through cell stress, transcription factor activation, and cytoskeleton reorganization. Each cassette has a 3 X 3 matrix of square openings, and a total of nine domains. Three different cell types are each arrayed in three of the domains, with the cells in a single domain expressing a single toxin biosensor. Each cell type expresses a different toxin biosensor in each of the domains in which it is arrayed.

Cell Types:

(A) SD3T3: connective tissue cell type: Serum-deprived 3T3 fibroblast (ATCC CCL-92)

(B) SNB19: neuronal tissue cell type: Human glioblastoma (ATCC#CRL-2219)

(C) RAW: Immune specific cell type: Murine macrophage cell line (ATCC#TIB-71)

Column 1 (A1/B1/C1) will provide a "low resolution" image of the sample. If the sample contains the minimum detectable concentration of either SEB, Cholera Tox, or Bot. Tox, it will elicit a positive cell stress response from each of the 3 tissue specific cells. A positive response

in Column 1 would entail a "higher resolution" probe of the sample in Columns 2 and 3. The modality of action and tissue specificity of the toxin are dissected in Columns 2 and 3, using the NF-kB and β-tubulin biosensors.

Table 6. Matrix 2: Dissection of pathway and organ localization of 1 toxin per cassette plus an internal control

	Toxin 1	Toxin 1	PBS
	1	2	3
A	SD3T3 (HSP)	SD3T3 (NF-kB + β-tubulin)	SD3T3 (NF-kB + β-tubulin)
B	SNB19 (HSP)	SNB19 (NF-kB + β-tubulin)	SNB19 (NF-kB + β-tubulin)
C	RAW (HSP)	RAW (NF-kB + β-tubulin)	RAW (NF-kB + β-tubulin)

Each cassette with a 3 X 3 array of the 3 cell types with each of the 3 toxin biosensors enables addressing the effect of a single toxin on connective tissue, neuronal tissue, and immune specific cells as measured through cell stress, transcription factor activation, and cytoskeleton reorganization. Columns 2 and 3 have cells expressing 2 fluorescent protein biosensors each. This enables simultaneous detection of 2 different sets of macromolecules representing two different pathways within the same cells.

Table 7. Matrix 3: Dissection of pathway and organ localization of 3 toxins per cassette

	Toxin 1	Toxin 2	Toxin 3
	1	2	3
A	SD3T3 (HSP + NF-kB + β-tubulin)	SD3T3 (HSP + NF-kB + β-tubulin)	SD3T3 (HSP + NF-kB + β-tubulin)
B	SNB19 (HSP + NF-kB + β-tubulin)	SNB19 (HSP + NF-kB + β-tubulin)	SNB19 (HSP + NF-kB + β-tubulin)
C	RAW (HSP + NF-kB + β-tubulin)	RAW (HSP + NF-kB + β-tubulin)	RAW (HSP + NF-kB + β-tubulin)

Each cell on the substrate has the capacity to simultaneously report the effect of a toxin on cell stress, transcription factor activation and translocation, and reorganization of the cytoskeletal proteins.

Experimental design and results of 3 x 3 matrix model system

The following matrix of toxins, analogs, and assays were used for 3 x 3 matrix study based on the above examples, except that the Swiss 3T3 cells were exposed to the toxin analogs for 45 minutes. In each assay, the analogs were used at the following concentrations:

Medium exchange	negative control
TNF α	1.8 nM
Anisomycin	30 μM
Cytochalasin D	2 μM

Cell Stress Target

Quantitative results were determined based on data obtained from 1500 cells totaled across 16 wells. The proportion of NF-kB protein in the nucleus versus the cytoplasm for wells treated by the toxin analogs is shown in **Figure 47**. The dominant response was obtained for TNF α.

SEB/T2 Target

Quantitative results were determined based on data obtained from 1500 cells totaled across 16 wells. Phosphorylated p38 MAPK in the nucleus versus the cytoplasm for wells treated by the toxin analogs was as shown in **Figure 48.** The dominant response was obtained for anisomycin.

Botulinum C2 Target

Quantitative results were obtained based on results from 500 cells totaled across 6 wells. Cell area for cells treated with the toxin analogs was as shown in **Figure 49**. The dominant response was obtained for cytochalasin D.

The present invention is not limited by the aforementioned particular preferred embodiments. It will occur to those ordinarily skilled in the art that various modifications may be made to the disclosed preferred embodiments without diverting from the concept of the invention. All such modifications are intended to be within the scope of the present invention.

We claim

1. A cassette for cell screening, comprising

a) a substrate having a surface;

b) a fluid delivery system mated with the substrate, wherein the fluid delivery system comprises:

1) a three by three matrix of openings or depressions, wherein each region of the substrate enclosed by the opening or depression in the matrix comprises an individually addressable domain; and

2) microfluidic channels that supply fluid to the domains.

2. The cassette of claim 1, wherein each domain comprises cell binding sites separated by cell repulsive regions.

3. The cassette of claim 2, wherein each domain comprises a single cell type arrayed on the cell binding sites.

4. The cassette of claim 3 wherein the cell expresses one or more biosensors.

5. The cassette of claim 4 wherein at least three different cell types are arrayed on the surface.

6. The method of claim 5 wherein each of the three different cell types are specific for different tissue types.

7. The cassette of claim 6 wherein the three different tissue types are connective tissue, neuronal tissue, and immune system.

8. A method for cell screening, comprising:

a) providing the cassette for cell screening of any of claims 3-7;

b) providing an optical system to obtain images of the cells;

c) contacting the domains with a test compound

d) obtaining images of the cells to determine an effect of the test compound on the cells.

9. A method for detecting a toxin pathway and organ localization, comprising

a) providing the cassette for cell screening of claim 5-7

b) providing an optical system to obtain images of the cells;

c) contacting the domains with a test sample potentially comprising a toxin;

d) obtaining images of the cells to determine an effect of the toxin on the cells, wherein the effect of the toxin on the cells indicates the toxin pathway and organ localization.

10. The method of claim 9, wherein the one or more biosensors expressed by the cells are selected from the group consisting of detectors, classifiers, and identifiers.

11. An automated method for cell based toxin detection and organ localization comprising

-providing an array of locations containing cells to be treated with a test substance, wherein the array comprises at least a first cell type and a second cell type, and wherein the first cell type and the second cell type are not contained on the same location in the array; wherein the first and second cell types are derived from different organ types; wherein each of the cell types comprises at least one luminescent reporter molecule; wherein the localization, distribution, structure, or activity of the at least one luminescent reporter molecule is altered by a toxin to be detected;

-contacting the at least first cell type and second cell type with the test substance either before or after possession of the at least one luminescent reporter molecules by the first cell type and the second cell type;

-imaging or scanning multiple cells in each of the locations containing the first cell type or the second cell type to obtain luminescent signals from the luminescent reporter molecule in the first cell type and the second cell type;

-converting the luminescent signals into digital data;

-utilizing the digital data to automatically measure the localization, distribution, structure or activity of the at least one luminescent reporter molecule on or in the first cell type and the second cell type, wherein a change in the localization, distribution, structure or activity of the luminescent reporter molecule indicates the presence of a toxin and an organ localization of the toxin.

12. The method of claim 11, wherein the array further comprises a third cell type, wherein the first, second, and third cell types are not contained on the same location in the array; and wherein the first, second, and third cell types are derived from different organ types.

13. The method of claim 11 or 12 wherein one or more of the cell types further comprises at least a second luminescent reporter molecule; wherein the localization, distribution, structure, or activity of the second luminescent reporter molecule is altered by a toxin to be detected.

14. The method of claim 13 wherein one or more of the cell types further comprises at least a third luminescent reporter molecule; wherein the localization, distribution, structure, or activity of the third luminescent reporter molecule is altered by a toxin to be detected.